

FORM PTO-1390 (REV. 9-2001)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 10447-22	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	
				10/089058	
INTERNATIONAL APPLICATION NO. PCT/CA00/01162		INTERNATIONAL FILING DATE October 4, 2000		PRIORITY DATE CLAIMED October 4, 1999 and April 14, 2000	
TITLE OF INVENTION IMPROVED RICIN-LIKE TOXINS FOR TREATMENT OF CANCER					
APPLICANT(S) FOR DO/EO/US CURTIS BRAUN, ADMIR PURAC, THOR BORGFORD					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<ol style="list-style-type: none">1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))<ol style="list-style-type: none">a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).<ol style="list-style-type: none">a. <input type="checkbox"/> is attached hereto.b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))<ol style="list-style-type: none">a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).b. <input type="checkbox"/> have been communicated by the International Bureau.c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.d. <input type="checkbox"/> have not been made and will not be made.8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). <p>Items 11 to 20 below concern document(s) or information included:</p> <ol style="list-style-type: none">11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.15. <input type="checkbox"/> A substitute specification.16. <input type="checkbox"/> A change of power of attorney and/or address letter.17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).20. <input type="checkbox"/> Other items or information:					

U.S. APPLICATION NO. (if known, see 37 CFR 1.53) 10/089058		INTERNATIONAL APPLICATION NO. PCT/CA00/01162		ATTORNEY'S DOCKET NUMBER 10447-22	
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<p>21. <input checked="" type="checkbox"/> The following fees are submitted:</p> <p>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):</p> <p>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00</p> <p style="text-align: right;">ENTER APPROPRIATE BASIC FEE AMOUNT =</p>				CALCULATIONS PTO USE ONLY	
<p>Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).</p>				<p>\$ 890.00</p>	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$
Total claims	- 20 =		x \$18.00	\$
Independent claims	- 3 =		x \$84.00	\$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$280.00	\$
TOTAL OF ABOVE CALCULATIONS =				\$ 890.00
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				+
SUBTOTAL =				\$ 890.00
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$
TOTAL NATIONAL FEE =				\$ 890.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$
TOTAL FEES ENCLOSED =				\$ 890.00
				Amount to be refunded:
				\$
				charged:
				\$

a. ☒ A check in the amount of \$ **890.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **022095**. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Patent Application Data Sheet

Application Information

Application Type:: Regular

Subject Matter:: Utility

Suggested
Classification::

Suggested Group Art
Unit::

CD-ROM or CD-R?: None

Number of CD disks:: 0

Number of copies of CDs:: 0

Sequence submission?: YES

Computer Readable
Form (CRF)?:: YES

Number of copies of CRF:: 1

Title:: IMPROVED RICIN-LIKE TOXINS FOR
TREATMENT OF CANCER

Attorney Docket Number:: 10447-22

Request for Early
Publication?: NO

Request for Non-Publication?: NO

Suggested Drawing Figure::

Total Drawing Sheets:: 94

Small Entity?: YES

Latin Name::

Variety denomination

name::

Petition included?:: No

Petition Type::

Licensed US Govt.
Agency::

Contract or Grant
Numbers::

Secrecy Order in
Parent Appl.?:: No

Applicant Information

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Barristers and Solicitors/Patent and Trade Mark Agents
Practice Restricted to Intellectual Property Law

April 3, 2002

Micheline Gravelle B.Sc., M.Sc. (Immunol.)
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Your Reference: n/a
Our Reference: 10447-22

Commissioner for Patents and Trademarks
Washington, D.C. 20231
U.S.A.

Dear Sirs:

Re: PRELIMINARY AMENDMENT
United States National Phase Entry of PCT/CA00/01162
Entitled: IMPROVED RICIN-LIKE TOXINS FOR TREATMENT OF CANCER
Inventors: Curtis Braun, Admir Purac and Thor Borgford

We are simultaneously entering national phase in the United States for PCT/CA01/01162. The present letter is to file a Preliminary Amendment to the application. Please amend the application as follows:

In the Claims:

Please amend claims 3, 5, 7, 8, 9, 10, 11, 12, 15, 17, 19, 20, 24, 26, 28, 29, 30, 33, 35, 37, 38, 39 and 40 as follows:

3. (Amended) A nucleic acid molecule according to claim 1 wherein the protease is associated with a cancer cell.

5. (Amended) A nucleic acid molecule according to claim 1 wherein the protease is associated with an inflammatory cell.

7. (Amended) A nucleic acid molecule of claim 1 wherein the A chain is ricin A chain, abrin toxin A chain, diphtheria toxin A chain, Domain III of Pseudomonas

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exotoxin, volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain, viscumin toxin A chain or shiga toxin A chain.

8. (Amended) A nucleic acid molecule of claim 1 wherein the B chain is ricin B chain, abrin toxin B chain, diphtheria toxin B chain, Domain I/II of *Pseudomonas* exotoxin, volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain, viscumin toxin B chain or shiga toxin B chain.

9. (Amended) A nucleic acid molecule according to claim 1 having a nucleic acid sequence selected from the group consisting of the nucleic acid sequence of pAP301 as shown in Figure 1B; the nucleic acid sequence of pAP302 as shown in Figure 2B; the nucleic acid sequence of pAP303 as shown in Figure 3B; the nucleic acid sequence of pAP304 as shown in Figure 4B; the nucleic acid sequence of pAP305 as shown in Figure 5B; the nucleic acid sequence of pAP308 as shown in Figure 6B; the nucleic acid sequence of pAP309 as shown in Figure 7B; the nucleic acid sequence of pAP313 as shown in Figure 8B; the nucleic acid sequence of pAP314 as shown in Figure 9B; the nucleic acid sequence of pAP315 as shown in Figure 10B; the nucleic acid sequence of pAP316 as shown in Figure 11B; the nucleic acid sequence of pAP318 as shown in Figure 12B; the nucleic acid sequence of pAP320 as shown in Figure 13B; the nucleic acid sequence of pAP321 as shown in Figure 14B; the nucleic acid sequence of pAP322 as shown in Figure 15B; the nucleic acid sequence of pAP323 as shown in Figure 16B; the nucleic acid sequence of pAP324 as shown in Figure 17B; and the nucleic acid sequence of pAP325 as shown in Figure 18B.

10. (Amended) A nucleic acid molecule according to claim 1 wherein the nucleotide sequence of the linker is selected from the group consisting of: the nucleic acid sequence of pAP301 as shown in Figure 1A; the nucleic acid sequence of pAP302 as shown in Figure 2A; the nucleic acid sequence of pAP303 as shown in Figure 3A; the nucleic acid sequence of pAP304 as shown in Figure 4A; the nucleic acid sequence

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of pAP305 as shown in Figure 5A; the nucleic acid sequence of pAP308 as shown in Figure 6A; the nucleic acid sequence of pAP309 as shown in Figure 7A; the nucleic acid sequence of pAP313 as shown in Figure 8A; the nucleic acid sequence of pAP314 as shown in Figure 9A; the nucleic acid sequence of pAP315 as shown in Figure 10A; the nucleic acid sequence of pAP316 as shown in Figure 11A; the nucleic acid sequence of pAP318 as shown in Figure 12A; the nucleic acid sequence of pAP320 as shown in Figure 13A; the nucleic acid sequence of pAP321 as shown in Figure 14A; the nucleic acid sequence of pAP322 as shown in Figure 15A; the nucleic acid sequence of pAP323 as shown in Figure 16A; the nucleic acid sequence of pAP324 as shown in Figure 17A; and the nucleic acid sequence of pAP325 as shown in Figure 18A.

11. (Amended) A plasmid incorporating the nucleic acid molecule of claim 1.
12. (Amended) A baculovirus transfer vector incorporating the nucleic acid molecule according to claim 1.
15. (Amended) A protein according to claim 13 wherein the protease is associated with a cancer cell.
17. (Amended) A protein according to claim 13 wherein the protease is associated with an inflammatory cell.
19. (Amended) A recombinant protein of claim 13 wherein the A chain is ricin A chain, abrin toxin A chain, diphtheria toxin A chain, Domain III of Pseudomonas exotoxin, volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain, viscumin toxin A chain, or shiga toxin A chain.
20. (Amended) A recombinant protein of claim 13 wherein the B chain is ricin B chain, abrin toxin B chain, diphtheria toxin B chain, Domain I/II of Pseudomonas

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exotoxin, volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain, viscumin toxin B chain, or shiga toxin B chain.

24. (Amended) A method according to claim 22 wherein the protease is associated with a cancer cell.

26. (Amended) A method according to claim 22 wherein the protease is associated with an inflammatory cell.

28. (Amended) A method of inhibiting or destroying cells having a specific protease comprising contacting the cells with an effective amount a recombinant protein according to claim 13.

29. (Amended) A method of treating a cell having a specific protease comprising administering an effective amount of a recombinant protein according to claim 13 to an animal in need thereof.

30. (Amended) A method of treating a cell having a specific protease comprising administering an effective amount of a nucleic acid molecule according to claim 1 to an animal in need thereof.

33. (Amended) A process according to claim 31 wherein the protease is associated with a cancer cell.

35. (Amended) A process according to claim 31 wherein the protease is associated with an inflammatory cell.

37. (Amended) A pharmaceutical composition for treating cancer comprising a recombinant protein of claim 13 and a pharmaceutically acceptable carrier, diluent or excipient.

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38. (Amended) A pharmaceutical composition for treating inflammation comprising a recombinant protein of claim 13 and a pharmaceutically acceptable carrier, diluent or excipient.

39. (Amended) A pharmaceutical composition for treating a cell having a specific protease comprising a nucleic acid molecule of claim 1 and a pharmaceutically acceptable carrier, diluent or excipient.

40. (Amended) A pharmaceutical composition for treating a cell having a specific protease comprising an amino acid molecule of claim 1 and a pharmaceutically acceptable carrier, diluent or excipient.

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REMARKS

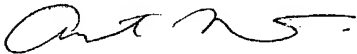
By the present amendment, claims 3, 5, 7, 8, 9, 10, 11, 12, 15, 17, 19, 20, 24, 26, 28, 29, 30, 33, 35, 37, 38, 39 and 40 have been amended in order to delete multiple dependencies.. The Preliminary Amendment does not contain new matter.

Entry of the above preliminary amendment is respectfully requested. Please calculate the claim fee for the application once the amendment has been entered.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "Version with markings to show changes made".

Respectfully submitted,

Curtis Braun, Amir Purac, Thor Borgford



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Version with markings to show changes made**In the Claims:**

Claims 3, 5, 7, 8, 9, 10, 11, 12, 15, 17, 19, 20, 24, 26, 28, 29, 30, 33, 35, 37, 38, 39 and 40 have been amended as follows:

3. (Amended) A nucleic acid molecule according to claim 1 [or 2] wherein the protease is associated with a cancer cell.

5. (Amended) A nucleic acid molecule according to claim 1 [or 2] wherein the protease is associated with an inflammatory cell.

7. (Amended) A nucleic acid molecule of [any one of] claim[s] 1[-6] wherein the A chain is ricin A chain, abrin toxin A chain, diphtheria toxin A chain, Domain III of Pseudomonas exotoxin, volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain, viscumin toxin A chain or shiga toxin A chain.

8. (Amended) A nucleic acid molecule of [any one of] claim[s] 1[-6] wherein the B chain is ricin B chain, abrin toxin B chain, diphtheria toxin B chain, Domain I/II of Pseudomonas exotoxin, volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain, viscumin toxin B chain or shiga toxin B chain.

9. (Amended) A nucleic acid molecule according to [any one of] claim[s] 1 [to 8] having a nucleic acid sequence selected from the group consisting of the nucleic acid sequence of pAP301 as shown in Figure 1B; the nucleic acid sequence of pAP302 as shown in Figure 2B; the nucleic acid sequence of pAP303 as shown in Figure 3B; the nucleic acid sequence of pAP304 as shown in Figure 4B; the nucleic acid sequence of pAP305 as shown in Figure 5B; the nucleic acid sequence of pAP308 as shown in Figure 6B; the nucleic acid sequence of pAP309 as shown in Figure 7B; the nucleic acid sequence of pAP313 as shown in Figure 8B; the nucleic acid sequence of pAP314 as shown in Figure 9B; the nucleic acid sequence of pAP315 as shown in Figure 10B; the nucleic acid sequence of pAP316 as shown in Figure 11B; the nucleic acid sequence of pAP318 as shown in Figure 12B; the nucleic acid sequence of pAP320 as shown in Figure 13B; the nucleic acid sequence of pAP321 as shown in Figure 14B; the nucleic acid sequence of pAP322 as shown in Figure 15B; the nucleic acid sequence of pAP323 as shown in Figure 16B; the nucleic acid sequence of pAP324 as shown in Figure 17B; and the nucleic acid sequence of pAP325 as shown in Figure 18B.

10. (Amended) A nucleic acid molecule according to [any one of] claim[s] 1 [to 8] wherein the nucleotide sequence of the linker is selected from the group consisting of: the nucleic acid sequence of pAP301 as shown in Figure 1A; the nucleic acid sequence of pAP302 as shown in Figure 2A; the nucleic acid sequence of pAP303 as

shown in Figure 3A; the nucleic acid sequence of pAP304 as shown in Figure 4A; the nucleic acid sequence of pAP305 as shown in Figure 5A; the nucleic acid sequence of pAP308 as shown in Figure 6A; the nucleic acid sequence of pAP309 as shown in Figure 7A; the nucleic acid sequence of pAP313 as shown in Figure 8A; the nucleic acid sequence of pAP314 as shown in Figure 9A; the nucleic acid sequence of pAP315 as shown in Figure 10A; the nucleic acid sequence of pAP316 as shown in Figure 11A; the nucleic acid sequence of pAP318 as shown in Figure 12A; the nucleic acid sequence of pAP320 as shown in Figure 13A; the nucleic acid sequence of pAP321 as shown in Figure 14A; the nucleic acid sequence of pAP322 as shown in Figure 15A; the nucleic acid sequence of pAP323 as shown in Figure 16A; the nucleic acid sequence of pAP324 as shown in Figure 17A; and the nucleic acid sequence of pAP325 as shown in Figure 18A.

11. (Amended) A plasmid incorporating the nucleic acid molecule of [any one of] claim[s] 1 [to 10].

12. (Amended) A baculovirus transfer vector incorporating the nucleic acid molecule according to [any one of] claim[s] 1 [to 10].

15. (Amended) A protein according to claim 13 [or 14] wherein the protease is associated with a cancer cell.

17. (Amended) A protein according to claim 13 [or 14] wherein the protease is associated with an inflammatory cell.

19. (Amended) A recombinant protein of [anyone of] claim[s] 13[-18] wherein the A chain is ricin A chain, abrin toxin A chain, diphtheria toxin A chain, Domain III of Pseudomonas exotoxin, volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain, viscumin toxin A chain, or shiga toxin A chain.

20. (Amended) A recombinant protein of [anyone of] claim[s] 13[-18] wherein the B chain is ricin B chain, abrin toxin B chain, diphtheria toxin B chain, Domain I/II of Pseudomonas exotoxin, volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain, viscumin toxin B chain, or shiga toxin B chain.

24. (Amended) A method according to claim 22 [or 23] wherein the protease is associated with a cancer cell.

26. (Amended) A method according to claim 22 [or 23] wherein the protease is associated with an inflammatory cell.

28. (Amended) A method of inhibiting or destroying cells having a specific protease comprising contacting the cells with an effective amount a recombinant protein according to [any one of] claim[s] 13 [to 21].

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29. (Amended) A method of treating a cell having a specific protease comprising administering an effective amount of a recombinant protein according to [any one of] claim[s] 13 [to 21] to an animal in need thereof.

30. (Amended) A method of treating a cell having a specific protease comprising administering an effective amount of a nucleic acid molecule according to [any one of] claim[s] 1 [to 10] to an animal in need thereof.

33. (Amended) A process according to claim 31 [or 32] wherein the protease is associated with a cancer cell.

35. (Amended) A process according to claim 31 [or 32] wherein the protease is associated with an inflammatory cell.

37. (Amended) A pharmaceutical composition for treating cancer comprising a recombinant protein of [any one of] claim[s] 13 [to 21] and a pharmaceutically acceptable carrier, diluent or excipient.

38. (Amended) A pharmaceutical composition for treating inflammation comprising a recombinant protein of [any one of] claim[s] 13 [to 21] and a pharmaceutically acceptable carrier, diluent or excipient.

39. (Amended) A pharmaceutical composition for treating a cell having a specific protease comprising a nucleic acid molecule of [any one of] claim[s] 1 [to 10] and a pharmaceutically acceptable carrier, diluent or excipient.

40. (Amended) A pharmaceutical composition for treating a cell having a specific protease comprising an amino acid molecule of [any one of] claim[s] 1 [to 10] and a pharmaceutically acceptable carrier, diluent or excipient.

Title: IMPROVED RICIN-LIKE TOXINS FOR TREATMENT OF CANCER

FIELD OF THE INVENTION

The invention relates to proteins useful as therapeutics against unhealthy cells such as those which occur in inflammation and cancer. The proteins contain A and B chains of a ricin-like toxin linked by a novel linker sequence that is specifically cleaved and activated by proteases specific to cancer.

BACKGROUND OF THE INVENTION

Bacteria and plants are known to produce cytotoxic proteins which may consist of one, two or several polypeptides or subunits. Those proteins having a single subunit may be loosely classified as Type I proteins. Many of the cytotoxins which have evolved two subunit structures are referred to as type II proteins (Saelinger, C.B. in Trafficking of Bacterial Toxins (eds. Saelinger, C.B.) 1-13 (CRC Press Inc., Boca Raton, Florida, 1990). One subunit, the A chain, possesses the toxic activity whereas the second subunit, the B chain, binds cell surfaces and mediates entry of the toxin into a target cell. A subset of these toxins kill target cells by inhibiting protein biosynthesis. For example, bacterial toxins such as diphtheria toxin or Pseudomonas exotoxin inhibit protein synthesis by inactivating elongation factor 2. Plant toxins such as ricin, abrin, and bacterial toxin Shiga toxin, inhibit protein synthesis by directly inactivating the ribosomes (Olsnes, S. & Phil, A. in Molecular action of toxins and viruses (eds. Cohen, P. & vanHeyningen, S.) 51-105 Elsevier Biomedical Press, Amsterdam, 1982).

Ricin, derived from the seeds of *Ricinus communis* (castor oil plant), may be the most potent of the plant toxins. It is estimated that a single ricin A chain is able to inactivate ribosomes at a rate of 1500 ribosomes/minute. Consequently, a single molecule of ricin is enough to kill a cell (Olsnes, S. & Phil, A. in Molecular action of toxins and viruses (eds. Cohen, P. & vanHeyningen, S.) (Elsevier Biomedical Press, Amsterdam, 1982). The ricin toxin is a glycosylated heterodimer consisting of A and B chains with molecular masses of 30,625 Da and 31,431 Da linked by a disulphide bond. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y. & Tsurugi, K. J., *Biol. Chem.* 262:8128 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al., *Biol. Chem.* 261:7912 (1986)). Once the toxin molecule consisting of the A and B chains is internalized into the cell via clathrin-dependent or independent mechanisms, the greater reduction potential within the cell induces a release of the active A chain, eliciting its inhibitory effect on protein synthesis and its cytotoxicity (Emmanuel, F. et al., *Anal. Biochem.* 173: 134-141 (1988); Blum, J.S. et al., *J. Biol. Chem.* 266: 22091-22095 (1991); Fiani, M.L. et al., *Arch. Biochem. Biophys.* 307: 225-230 (1993)). Empirical

evidence suggests that activated toxin (e.g. ricin, shiga toxin and others) in the endosomes is transcytosed through the trans-Golgi network to the endoplasmic reticulum by retrograde transport before the A chain is translocated into the cytoplasm to elicit its action (Sandvig, K. & van Deurs, B., *FEBS Lett.* 346: 99-102 (1994)).

5 Protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (preproricin) with an amino acid N-terminal presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M., *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M., *Eur. J. Biochem.* 146:411-416
10 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and B chains (Lord, J.M. et al., *FASAB journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is stored in protein bodies
15 inside the plant cells. The A chain is inactive in proricin (O'Hare, M. et al., *FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin (Richardson, P.T. et al., *FEBS Lett.* 255:15-20 (1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell. The
20 exact mechanism of A chain release and activation in target cell cytoplasm is not known (Lord, J.M. et al., *FASAB journal* 8:201-208 (1994)). However, it is known that for activation to take place the disulfide bond between the A and B chains must be reduced and, hence, the linkage between subunits broken.

Diphtheria toxin is produced by *Corynebacterium diphtheriae* as a 535 amino
25 acid polypeptide with a molecular weight of approximately 58kD (Greenfield, L. et al., *Proc. Natl. Acad. Sci. USA* 80:6853-6857 (1983); Pastan, I. et al., *Annu. Rev. Biochem.* 61:331-354 (1992); Collier, R.J. & Kandel, J., 1. *Biol. Chem.* 246:1496-1503 (1971)). It is secreted as a single-chain polypeptide consisting of 2 functional domains. Similar to proricin, the N-terminal domain (A-chain) contains the cytotoxic moiety whereas the C-
30 terminal domain (B-chain) is responsible for binding to the cells and facilitates toxin endocytosis. Conversely, the mechanism of cytotoxicity for diphtheria toxin is based on ADP-ribosylation of EF-2 thereby blocking protein synthesis and producing cell death. The 2 functional domains in diphtheria toxin are linked by an arginine-rich peptide sequence as well as a disulphide bond. Once the diphtheria toxin is internalized into the
35 cell, the arginine-rich peptide linker is cleaved by trypsin-like enzymes and the disulphide bond (Cys 186-201) is reduced. The cytotoxic domain is subsequently translocated into the cytosol substantially as described above for ricin and elicits ribosomal inhibition and cytotoxicity.

Pseudomonas exotoxin is also a 66kD single-chain toxin protein secreted by *Pseudomonas aeruginosa* with a similar mechanism of cytotoxicity to that of diphtheria toxin (Pastan, I. et al., *Annu. Rev. Biochem.* 61:331-354 (1992); Ogata, M. et al., *J. Biol. Chem.* 267:25396-25401 (1992); Vagil, M.L. et al., *Infect. Immunol.* 16:353-361 (1977)).

5 *Pseudomonas* exotoxin consists of 3 conjoint functional domains. The first domain I (amino acids 1-252) is responsible for cell binding and toxin endocytosis, a second domain II (amino acids 253-364) is responsible for toxin translocation from the endocytic vesicle to the cytosol, and a third domain III (amino acids 400-613) is responsible for protein synthesis inhibition and cytotoxicity. After *Pseudomonas* exotoxin enters the cell, the
10 liberation of the cytotoxic domain is effected by both proteolytic cleavage of a polypeptide sequence in the second domain (near Arg 279) and the reduction of the disulphide bond (Cys 265-287) in the endocytic vesicles. In essence, the overall pathway to cytotoxicity is analogous to diphtheria toxin with the exception that the toxin translocation domain in *Pseudomonas* exotoxin is structurally distinct.

15 Class 2 ribosomal inhibitory proteins (RIP-2) constitute other toxins possessing distinct functional domains for cytotoxicity and cell binding/toxin translocation which include abrin, modeccin, volkensin, (Sandvig, K. et al., *Biochem. Soc. Trans.* 21:707-711 (1993)) and mistle toe lectin (viscumin) (Olsnes, S. & Phil, A. in *Molecular action of toxins and viruses* (eds. Cohen, P. & vanHeyningen, S.) 51-105
20 Elsevier Biomedical Press, Amsterdam, 1982; Fodstad, et al. *Canc. Res.* 44: 862 (1984)). Some toxins such as Shiga toxin and cholera toxin also have multiple polypeptide chains responsible for receptor binding and endocytosis.

The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains have been described (Rutenber, E. et al. *Proteins* 10:240-250 (1991);
25 Weston et al., *Mol. Bio.* 244:410-422,1994; Lamb and Lord, *Eur. J. Biochem.* 14:265 (1985); Halling, K. et al. *Nucleic Acids Res.* 13:8019 (1985)). Similarly, the genes for diphtheria toxin and *Pseudomonas* exotoxin have been cloned and sequenced, and the 3-dimensional structures of the toxin proteins have been elucidated and described (Columblatti, M. et al., *J. Biol. Chem.* 261:3030-3035 (1986); Allured, V.S. et al., *Proc. Natl. Acad. Sci. USA*
30 83:1320-1324 (1986); Gray, G.L. et al., *Proc. Natl. Acad. Sci. USA* 81:2645-2649 (1984); Greenfield, L. et al., *Proc. Natl. Acad. Sci. USA* 80:6853-6857 (1983); Collier, R.J. et al., *J. Biol. Chem.* 257:5283-5285 (1982)).

The potential of bacterial and plant toxins for inhibiting mammalian retroviruses, particularly acquired immunodeficiency syndrome (AIDS), has been
35 investigated. Bacterial toxins such as *Pseudomonas* exotoxin and subunit A of diphtheria toxin; dual chain ribosomal inhibitory plant toxins such as ricin, and single chain ribosomal inhibitory proteins such as trichosanthin and pokeweed antiviral protein have been used for the elimination of HIV infected cells (Olson et al., *AIDS Res. and*

Human Retroviruses 7:1025-1030 (1991)). The high toxicity of these toxins for mammalian cells, combined with a lack of specificity of action poses a major problem to the development of pharmaceuticals incorporating the toxins, such as immunotoxins.

Due to their extreme toxicity there has been much interest in making ricin-based immunotoxins as therapeutic agents for specifically destroying or inhibiting infected or tumourous cells or tissues (Vitetta et al., *Science* 238:1098-1104(1987)). An immunotoxin is a conjugate of a specific cell binding component, such as a monoclonal antibody or growth factor and the toxin in which the two protein components are covalently linked. Generally, the components are chemically coupled. However, the linkage may also be a peptide or disulfide bond. The antibody directs the toxin to cell types presenting a specific antigen thereby providing a specificity of action not possible with the natural toxin. Immunotoxins have been made both with the entire ricin molecule (i.e. both chains) and with the ricin A chain alone (Spooner et al., *Mol. Immunol.* 31:117-125, (1994)).

Immunotoxins made with the ricin dimer (IT-Rs) are more potent toxins than those made with only the A chain (IT-As). The increased toxicity of IT-Rs is thought to be attributed to the dual role of the B chains in binding to the cell surface and in translocating the A chain to the cytosolic compartment of the target cell (Vitetta et al., *Science* 238:1098-1104 (1987); Vitetta & Thorpe, *Seminars in Cell Biology* 2:47-58 (1991)). However, the presence of the B chain in these conjugates also promotes the entry of the immunotoxin into nontarget cells. Even small amounts of B chain may override the specificity of the cell-binding component as the B chain will bind nonspecifically to galactose associated with N-linked carbohydrates, which is present on most cells. IT-As are more specific and safer to use than IT-Rs. However, in the absence of the B chain the A chain has greatly reduced toxicity. Due to the reduced potency of IT-As as compared to IT-Rs, large doses of IT-As must be administered to patients. The large doses frequently cause immune responses and production of neutralizing antibodies in patients (Vitetta et al., *Science* 238:1098-1104 (1987)). IT-As and IT-Rs both suffer from reduced toxicity as the A chain is not released from the conjugate into the target cell cytoplasm.

A number of immunotoxins have been designed to recognize antigens on the surfaces of tumour cells and cells of the immune system (Pastan et al., *Annals New York Academy of Sciences* 758:345-353 (1995)). A major problem with the use of such immunotoxins is that the antibody component is its only targeting mechanism and the target antigen is often found on non-target cells (Vitetta et al., *Immunology Today* 14:252-259 (1993)). Also, the preparation of a suitable specific cell binding component may be problematic. For example, antigens specific for the target cell may not be available and many potential target cells and infective organisms can alter their antigenic make up rapidly to avoid immune recognition. In view of the extreme toxicity of proteins such as

ricin, the lack of specificity of the immunotoxins may severely limit their usefulness as therapeutics for the treatment of cancer and infectious diseases.

The insertion of intramolecular protease cleavage sites between the cytotoxic and cell-binding components of a toxin can mimic the way that the natural toxin is activated. European patent application no. 466,222 describes the use of maize-derived
5 pro-proteins which can be converted into active form by cleavage with extracellular blood enzymes such as factor, Xa, thrombin or collagenase. Garred, O. et al. (*J. Biol. Chem.* 270:10817-10821 (1995)) documented the use of a ubiquitous calcium-dependent serine protease, furin, to activate shiga toxin by cleavage of the trypsin-sensitive
10 linkage between the cytotoxic A-chain and the pentamer of cell-binding B-units. Westby et al. (*Bioconjugate Chem.* 3:375-381 (1992)) documented fusion proteins which have a specific cell binding component and proricin with a protease sensitive cleavage site specific for factor Xa within the linker sequence. O'Hare et al. (*FEBS Lett.* 273:200-204 (1990)) also described a recombinant fusion protein of RTA and staphylococcal protein A
15 joined by a trypsin-sensitive cleavage site. In view of the ubiquitous nature of the extracellular proteases utilized in these approaches, such artificial activation of the toxin precursor or immunotoxin does not confer a mechanism for intracellular toxin activation and the problems of target specificity and adverse immunological reactions to the cell-binding component of the immunotoxin remain.

20 In a variation of the approach of insertion of intramolecular protease cleavage sites on proteins which combine a binding chain and a toxic chain, Leppla, S.H. et al. (*Bacterial Protein Toxins zbl.bakt.suppl.* 24:431-442 (1994)) suggest the replacement of the native cleavage site of the protective antigen (PA) produced by *Bacillus anthracis* with a cleavage site that is recognized by cells that contain a particular protease. PA,
25 recognizes, binds, and thereby assists in the internalization of lethal factor (U) and edema toxin (ET), also produced by *Bacillus anthracis*. However, this approach is wholly dependent on the availability of LF, or ET and PA all being localized to cells wherein the modified PA can be activated by the specific protease. It does not confer a mechanism for intracellular toxin activation and presents a problem of ensuring sufficient
30 quantities of toxin for internalization in target cells.

The *in vitro* activation of a *Staphylococcus*-derived pore forming toxin, (α -hemolysin by extracellular tumour-associated proteases has been documented (Panchel, R.G. et al., *Nature Biotechnology* 14:852-857 (1996)). Artificial activation of α -hemolysin *in vitro* by said proteases was reported but the actual activity and utility of
35 α -hemolysin in the destruction of target cells were not demonstrated.

α -Hemolysin does not inhibit protein synthesis but is a heptameric transmembrane pore which acts as a channel to allow leakage of molecules up to 3 kD thereby disrupting the ionic balances of the living cell. The α -hemolysin activation

- 6 -

domain is likely located on the outside of the target cell (for activation by extracellular proteases). The triggering mechanism in the disclosed hemolysin precursor does not involve the intracellular proteolytic cleavage of 2 functionally distinct domains. Also, the proteases used for the α -hemolysin activation are ubiquitously secreted

5 extracellular proteases and toxin activation would not be confined to activation in the vicinity of diseased cells. Such widespread activation of the toxin does not confer target specificity and limits the usefulness of said α -hemolysin toxin as therapeutics due to systemic toxicity.

A variety of proteases specifically associated with malignancy have been

10 identified and described. For example, cathepsin is a family of serine, cysteine or aspartic endopeptidases and exopeptidases which has been implicated to play a primary role in cancer metastasis (Schwartz, M.K., Clin. Chim. Acta 237:67-78 (1995); Spiess, E. et al., J. Histochem. Cytochem. 42:917-929 (1-994); Scarborough, P.E. et al., Protein Sci. 2:264276 (1993); Sloane, B.F. et al., Proc. Natl. Acad. Sci. USA 83:2483- 2487

15 (1986); Mikkelsen, T. et al., J. Neurosurg 83:285-290 (1995)). Matrix metalloproteinases (MMPs or matrixins) are zinc-dependent proteinases consisting of collagenases, matrilysin, stromelysins, stromelysin-like MMPs, gelatinases, macrophage elastase, membrane-type MMPs (MT-MMPs) (Krane, S.M., Ann. N.Y. Acad. Sci. 732:1-10 (1994); Woessner, J.F., Ann. N.Y. Acad. Sci. 732:11-21 (1994); Carvalho, K. et al., Biochem.

20 Biophys. Res., Comm. 191:172-179 (1993); Nakano, A. et al. J. of Neurosurg, 83:298-307 (1995); Peng, K-W, et al. Human Gene Therapy, 8:729-738 (1997); More, D.H. et al. Gynaecologic oncology, 65:78-82 (1997), Ravanti, L., Kahari, V. Intl. J. Mol. Med. 6(4):391 (2000)). These proteases are involved in pathological matrix remodeling. Under normal

25 physiological conditions, regulation of matrixin activity is effected at the level of gene expression. Enzymatic activity is also controlled stringently by tissue inhibitors of metalloproteinases (TIMPs) (Murphy, G. et al., Ann. N.Y. Acad. Sci., 732:31-41 (1994)). The expression of MMP genes is reported to be activated in inflammatory disorders (e.g. rheumatoid arthritis) and malignancy.

The present inventors have prepared novel recombinant toxic proteins which

30 are specifically toxic to diseased cells but do not depend for their specificity of action on a specific cell binding component. The recombinant proteins toxins have an A chain of a ricin-like toxin linked to a B chain by a synthetic linker sequence which may be cleaved specifically by a protease localised in cells or tissues affected by a specific disease to liberate the toxic A chain thereby selectively inhibiting or destroying the diseased cells

35 or tissues (WO 98/49311 published November 5, 1998 which is incorporated herein by reference).

SUMMARY OF THE INVENTION

The present invention relates to novel linker sequences that can be used to prepare recombinant toxic proteins having an A chain of a ricin-like toxin linked to a B chain by the linker sequence. The novel linker sequences of the invention are illustrated in Figures 1-18.

5 In one aspect the present invention provides a purified and isolated nucleic acid encoding a linker sequence comprising: the nucleic acid sequence of pAP301 as shown in Figure 1A; the nucleic acid sequence of pAP302 as shown in Figure 2A; the nucleic acid sequence of pAP303 as shown in Figure 3A; the nucleic acid sequence of pAP304 as shown in Figure 4A; the nucleic acid sequence of pAP305 as shown in Figure 5A; the nucleic acid
10 sequence of pAP308 as shown in Figure 6A; the nucleic acid sequence of pAP309 as shown in Figure 7A; the nucleic acid sequence of pAP313 as shown in Figure 8A; the nucleic acid sequence of pAP314 as shown in Figure 9A; the nucleic acid sequence of pAP315 as shown in Figure 10A; the nucleic acid sequence of pAP316 as shown in Figure 11A; the nucleic acid sequence of pAP318 as shown in Figure 12A; the nucleic acid sequence of pAP320 as shown
15 in Figure 13A; the nucleic acid sequence of pAP321 as shown in Figure 14A; the nucleic acid sequence of pAP322 as shown in Figure 15A; the nucleic acid sequence of pAP323 as shown in Figure 16A; the nucleic acid sequence of pAP324 as shown in Figure 17A; and the nucleic acid sequence of pAP325 as shown in Figure 18A.

In another aspect, the present invention provides a purified and isolated
20 nucleic acid encoding a recombinant toxic protein comprising (a) a nucleotide sequence encoding an A chain of a ricin-like toxin, (b) a nucleotide sequence encoding a B chain of a ricin-like toxin and (c) a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence is not a native linker sequence of a ricin-like toxin, but rather a synthetic heterologous linker sequence containing a cleavage recognition site for a
25 specific protease. The A and or the B chain may be those of ricin. As used herein "specific protease" means a protease in any cell wherein there is expression of the protease at levels greater than those found in a corresponding healthy cell. Examples of a specific protease include MMPs, preferably MMP-2, MMP-9, MMP-14, and MT1-MMPs, and UPA, as well as others found in inflammatory cells and malignant cells. An
30 inflammatory cell includes any cell involved in the inflammation process having a specific protease.

The recombinant toxic proteins employing the novel linker sequences of the present invention may be used to treat various forms of cells having specific proteases such as inflammatory disorders including rheumatoid arthritis, atherosclerotic cells,
35 Crohn's disease, central nervous system disease as well as in cancer including, but not limited to, T- and B-cell lymphoproliferative diseases, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate, cancer and non small cell lung cancer. In an embodiment, of the invention

In a further aspect, the present invention provides a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a cancer-specific protease. The A and/or the B chain may be those of ricin. In an embodiment, the cleavage recognition site is the

cleavage recognition site for a cancer protease substantially as described above. In a particular embodiment, the cancer is T-cell or B-cell lymphoproliferative disease, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate cancer, non small cell lung cancer.

5 In a further aspect, the invention provides a pharmaceutical composition for treating a cell, such as an inflammatory cell or cancer cell, having a specific protease, comprising a recombinant protein of the invention and a pharmaceutically acceptable carrier, diluent or excipient.

10 In yet another aspect, the invention provides a method of inhibiting or destroying a cell having a specific protease, such as an inflammatory cell or a cancer cell, comprising the steps of preparing a recombinant protein of the invention having a heterologous linker sequence which contains a cleavage recognition site for the specific protease, and administering the recombinant protein to the cells. In an embodiment, the inflammatory state is rheumatoid arthritis, atherosclerotic cells, Crohn's disease, or
15 central nervous system disease. In another embodiment, the cancer is T-cell or B-cell lymphoproliferative disease, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate cancer, non small cell lung cancer.

20 The present invention also relates to a method of treating a cell having a specific protease such as an inflammatory cell or a cancer cell, wherein the cells affected by the condition and which have a specific protease, are treated by administering an effective amount of one or more recombinant proteins of the invention to an animal in need thereof.

25 Still further, a process is provided for preparing a pharmaceutical for treating a cell having a specific protease, such as an inflammatory cell or a cancer cell, wherein cells affected by condition have a specific protease, the steps for preparing the pharmaceutical comprising the steps of preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B
30 chains, wherein the linker sequence contains a cleavage recognition site for the specific protease; introducing the nucleic acid into a host cell; expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage recognition site for the
35 specific protease; and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the

detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

5 **DESCRIPTION OF THE DRAWINGS**

The invention will be better understood with reference to the drawings in which:

Figure 1A shows the nucleotide sequence of the MMP-9 linker region of pAP301;

10 Figure 1B shows the nucleotide sequence of the pAP301 insert containing ricin and the MMP-9 linker;

Figure 1C shows the amino acid sequence of the PAP301 linker and the wild type ricin linker;

15 Figure 2A shows the nucleotide sequence of the MMP-9 30 linker region of pAP302;

Figure 2B shows the nucleotide sequence of the pAP302 insert containing ricin and the MMP-9 linker;

Figure 2C shows the amino acid sequence of the PAP302 linker and the wild type ricin linker;

20 Figure 3A shows the nucleotide sequence of the MMP-9 linker region of pAP303;

Figure 3B shows the nucleotide sequence of the pAP303 insert containing ricin and the MMP-9 linker;

25 Figure 3C shows the amino acid sequence of the PAP303 linker and the wild type ricin linker;

Figure 4A shows the nucleotide sequence of the MMP-9 linker region of pAP304;

Figure 4B shows the nucleotide sequence of the pAP304 insert containing ricin and the MMP-9 linker;

30 Figure 4C shows the amino acid sequence of the PAP304 linker and the wild type ricin linker;

Figure 5A shows the nucleotide sequence of the MMP-9 linker region of pAP305;

35 Figure 5B shows the nucleotide sequence of the pAP305 insert containing ricin and the MMP-9 linker;

Figure 5C shows the amino acid sequence of the PAP305 linker and the wild type ricin linker;

Figure 6A shows the nucleotide sequence of the MMP-9 linker region of pAP308;

Figure 6B shows the nucleotide sequence of the pAP308 insert containing ricin and the MMP-9 linker;

5 Figure 6C shows the amino acid sequence of the pAP308 linker and the wild type ricin linker;

Figure 7A shows the nucleotide sequence of the MMP-9 linker region of pAP309;

10 Figure 7B shows the nucleotide sequence of the pAP309 insert containing ricin and the MMP-9 linker;

Figure 7C shows the amino acid sequence of the PAP309 linker and the wild type ricin linker;

Figure 8A shows the nucleotide sequence of the UPA linker region of pAP313;

15 Figure 8B shows the nucleotide sequence of the pAP313 insert containing ricin and the UPA linker;

Figure 8C shows the amino acid sequence of the PAP313 linker and the wild type ricin linker;

Figure 9A shows the nucleotide sequence of the UPA linker region of pAP314;

20 Figure 9B shows the nucleotide sequence of the pAP314 insert containing ricin and the UPA linker;

Figure 9C shows the amino acid sequence of the PAP314 linker and the wild type ricin linker;

Figure 10A shows the nucleotide sequence of the UPA linker region of pAP315;

25 Figure 10B shows the nucleotide sequence of the pAP315 insert containing ricin and the UPA linker;

Figure 10C shows the amino acid sequence of the PAP315 linker and the wild type ricin linker;

Figure 11A shows the nucleotide sequence of the MMP-9 linker region of pAP316;

30 Figure 11B shows the nucleotide sequence of the pAP316 insert containing ricin and the MMP-9 linker;

Figure 11C shows the amino acid sequence of the PAP316 linker and the wild type ricin linker;

35 Figure 12A shows the nucleotide sequence of the MMP-9 linker region of pAP318;

Figure 12B shows the nucleotide sequence of the pAP318 insert containing ricin and the MMP-9 linker;

Figure 19 shows the cleavage products of an MMP-9 digestion of PAP323, PAP324 and PAP325:

Figure 20 is a graph showing the treatment of human tumour A431 with PAP304;

Figure 21 is a graph showing the treatment of human tumour A431 with PAP305; and

5 Figure 22 is a graph showing a significant delay in tumor growth in the murine tumor model.

DETAILED DESCRIPTION OF THE INVENTION

1. Nucleic Acid Molecules of the Invention

As mentioned above, the present invention relates to isolated and purified
10 nucleic acid molecules encoding linker sequences. The present invention also relates to isolated and purified nucleic acid molecules encoding a recombinant toxic protein comprising (a) a nucleotide sequence encoding an A chain of a ricin-like toxin, (b) a nucleotide sequence encoding a B chain of a ricin-like toxin and (c) a nucleotide sequence encoding a linker amino acid sequence of the invention, linking the A and B chains. The
15 heterologous linker sequence contains a cleavage recognition site for a specific protease.

The term "isolated and purified" as used herein refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. An "isolated and purified" nucleic acid is also substantially free of
20 sequences which naturally flank the nucleic acid (i.e. sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

The term "linker sequence" as used herein refers to an internal amino acid
25 sequence within the protein encoded by a nucleic acid molecule of the invention which contains residues linking the A and B chain of a ricin-like toxin so as to render the A chain incapable of exerting its toxic effect, for example catalytically inhibiting translation of an eukaryotic ribosome. The linker sequences of the invention are heterologous to the A and B chain of a ricin-like toxin. By heterologous is meant that the
30 linker sequence is not a sequence native to the A or B chain of a ricin-like toxin or precursor thereof. However, preferably, the linker sequence may be of a similar length to the linker sequence of a ricin-like toxin and should not interfere with the role of the B chain in cell binding and transport into the cytoplasm. When the linker sequence is cleaved the A chain becomes active or toxic.

35 The nucleic acid molecule of the invention encoding a recombinant toxic protein is cloned by subjecting a preproricin cDNA clone to site-directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). Oligonucleotides, corresponding to the extreme 5' and 3' ends of

the preproricin gene are synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., *Eur. J. Biochem.* 145:266-270 (1985)), several oligonucleotide primers are designed to flank the start and stop codons of the preproricin open reading frame.

5 The preproricin cDNA is amplified using the upstream primer Ricin-99 or Ricin-109 and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). The purified PCR fragment encoding the preproricin cDNA is, then ligated into an Eco RV-
10 digested pBluescript 11 SK plasmid (Stratagene), and is used to transform competent XL1-Blue cells (Stratagene). The cloned PCR product containing the putative preproricin gene is confirmed by DNA sequencing of the entire cDNA clone.

 The preproricin cDNA clone is subjected to site directed mutagenesis; in order to generate a series of variants differing only in the sequence between the A and B chains
15 (linker region). The wild-type preproricin linker region is replaced with the heterogenous linker sequences that are cleaved by the various specific proteases.

 The linker regions of the variants encode a cleavage recognition sequence for a specific protease. The mutagenesis and cloning strategies used to generate a specific protease-sensitive linker variant are summarized in WO 98149311 to the present
20 inventor. Briefly, the first step involves a DNA amplification using a set of mutagenic primers in combination with the two flanking primers Ricin-109Eco and Ricin1729C PstI. Restriction digested PCR fragments are gel purified and then ligated with PVL1393 which has been digested with Eco RI and PstI. Ligation reactions are used to transform competent XLI-Blue cells (Stratagene). Recombinant clones are identified by restriction
25 digests of plasmid miniprep, DNA and the mutant linker sequences are confirmed by DNA sequencing.

 The nucleotide sequences of the novel linker sequences of the invention are as follows: the nucleic acid sequence of pAP301 is shown in Figure 1A; the nucleic acid sequence of pAP302 is shown in Figure 2A; the nucleic acid sequence of pAP303 is shown in
30 Figure 3A; the nucleic acid sequence of pAP304 is shown in Figure 4A; the nucleic acid sequence of pAP305 is shown in Figure 5A; the nucleic acid sequence of pAP308 is shown in Figure 6A; the nucleic acid sequence of pAP309 is shown in Figure 7A; the nucleic acid sequence of pAP313 is shown in Figure 8A; the nucleic acid sequence of pAP314 is shown in Figure 9A; the nucleic acid sequence of pAP315 is shown in Figure 10A; the nucleic acid
35 sequence of pAP316 is shown in Figure 11A; the nucleic acid sequence of pAP318 is shown in Figure 12A; the nucleic acid sequence of pAP320 is shown in Figure 13A; the nucleic acid sequence of pAP321 is shown in Figure 14A; the nucleic acid sequence of pAP322 is shown in Figure 15A; the nucleic acid sequence of pAP323 is shown in Figure 16A; the nucleic acid

sequence of pAP324 is shown in Figure 17A; and the nucleic acid sequence of pAP325 is shown in Figure 18A.

The nucleic acid molecule encoding a recombinant protein of the invention has sequences encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a specific protease as described above. The nucleotide sequences encoding the recombinant proteins of the invention are shown in Figures 1B-18B. The nucleic acid may be expressed to provide a recombinant protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a specific protease.

The nucleic acid molecule may comprise the A and/or B chain of ricin. The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains are published (Rutenber, E., et al. *Proteins* 10:240-250 (1991); Weston et al., *Mol. Biol.* 244:410-422 (1994); Lamb and Lord, *Eur. J. Biochem.* 14:265 (1985); Halling, K., et al., *Nucleic Acids Res.* 13:8019 (1985)). It will be appreciated that the invention includes nucleic acid molecules encoding truncations of A and B chains of ricin-like proteins and analogs and homologs of A and B chains of ricin-like proteins and truncations thereof (i.e., ricin-like proteins), as described herein. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a cDNA of the invention are encompassed by the invention.

Another aspect of the invention provides a nucleotide sequence which hybridizes under high stringency conditions to a nucleotide sequence encoding the A and/or B chains of a ricin-like protein. Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1 6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C may be employed.

The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C.

The nucleic acid molecule may comprise the A and/or B chain of a ricin-like toxin. Methods for cloning ricin-like toxins are known in the art and are described, for example, in E.P. 466,222. Sequences encoding ricin or ricin-like A and B chains may be obtained by selective amplification of a coding region, using sets of degenerative primers or probes for selectively amplifying the coding region in a genomic or cDNA library. Appropriate primers may be selected from the nucleic acid sequence of A and B chains of

The nucleic acid molecule of the invention may also encode a fusion protein. A
35 sequence encoding a heterologous linker sequence containing a cleavage recognition site for
a specific protease may be cloned from a cDNA or genomic library or chemically
synthesized based on the known sequence of such cleavage sites. The heterologous linker
sequence may then be fused in frame with the sequences encoding the A and B chains of

intracellularly thereby releasing the toxic A chain directly into the cytoplasm of the target cell. As a result, said cells are specifically targeted and normal cells are not directly exposed to the activated free A chain.

Ricin is a plant derived ribosome inhibiting protein which blocks protein
5 synthesis in eukaryotic cells. Ricin may be derived from the seeds of *Ricinus communis* (castor oil plant). The ricin toxin is a glycosylated heterodimer with A and B chain molecular masses of 30,625 Da and 31,431 Da respectively. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y; & Tsurugi, K. J. Biol. Chem. 262:8128 (1987)).
10 The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al., *Biol. Chem.* 261:7912 (1986)).

All protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (preproricin) with a 35 amino acid N-terminal presequence and 12 amino acid linker between the A and B chains. The pre-
15 sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M., *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M., *Eur. J. Biochem.* 146:411-416 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the
20 A and B chains (Lord, J.M. et al., *FASAB journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is stored in protein bodies inside plant cells. The A chain is inactive in the proricin (O'Hare, M., et al., *FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin
25 (Richardson, P.T. et al., *FEBS Lett.* 255:15-20 (1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell.

Ricin-like proteins include, but are not limited to, bacterial, fungal and plant toxins which have A and B chains and inactivate ribosomes and inhibit protein
30 synthesis. The A chain is an active polypeptide subunit which is responsible for the pharmacologic effect of the toxin. In most cases the active component of the A chain is an enzyme. The B chain is responsible for binding the toxin to the cell surface and is thought to facilitate entry of the A chain into the cell cytoplasm. The A and B chains in the mature toxins are linked by disulfide bonds. The toxins most similar in structure to ricin
35 are plant toxins which have one A chain and one B chain. Examples of such toxins include abrin which may be isolated from the seeds of *Abrus precatorius*, modeccin, volkensin and viscumin.

Ricin-like bacterial proteins include diphtheria toxin, which is produced by *Corynebacterium diphtheriae*, *Pseudomonas* exotoxin and cholera toxin. It will be appreciated that the term ricin-like toxins is also intended to include the A chain of those toxins which have only an A chain. The recombinant proteins of the invention could include the A chain of these toxins conjugated to, or expressed as, a recombinant protein with the B chain of another toxin. Examples of plant toxins having only an A chain include trichosanthin, MMC and pokeweed antiviral proteins, dianthin 30, dianthin 32, croton II, curcin 11 and wheat germ inhibitor. Examples of fungal toxins having only an A chain include alpha-sarcin, restrictocin, mitogillin, enomycin, phenomycin. Examples of bacterial toxins having only an A chain include cytotoxin from *Shigella dysenteriae* and related Shiga-like toxins. Recombinant trichosanthin and the coding sequence thereof is disclosed in U.S. Patents 5,101,025 and 5,128,460.

In addition to the entire A or B chains of a ricin-like toxin, it will be appreciated that the recombinant protein of the invention may contain only that portion of the A chain which is necessary for exerting its cytotoxic effect. For example, the first 30 amino acids of the ricin A chain may be removed resulting in a truncated A chain which retains toxic activity. The truncated ricin or ricin-like A chain may be prepared by expression of a truncated gene or by proteolytic degradation, for example with Nagarase (Funmatsu et al., *Jap. J. Med. Sci. Biol.* 23:264-267 (1970)). Similarly, the recombinant protein of the invention may contain only that portion of the B chain necessary for galactose recognition, cell binding and transport into the cell cytoplasm. Truncated B chains are described for example in E.P. 145,111. The A and B chains may be glycosylated or non-glycosylated. Glycosylated A and B chains may be obtained by expression in the appropriate host cell capable of glycosylation. Non-glycosylated chains may be obtained by expression in nonglycosylating host cells or by treatment to remove or destroy the carbohydrate moieties.

The proteins of the invention may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used. The expression vectors are "suitable for transformation of a host cell", which means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. Operatively linked is intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the inserted protein-sequence.

5 Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be
10 readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers,
15 and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary regulatory sequences may be supplied by the native A and B chains and/or its flanking regions.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or
20 transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, β -galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. Transcription of the selectable marker gene is monitored by changes in
25 the concentration of the selectable marker protein such as β -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. Cells that have incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for
30 expression of recombinant expression vectors of the invention and in particular to determine the effect of a mutation on expression and phenotype. It will be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a
35 fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow

separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMal (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E
5 binding protein, or protein A, respectively, to the recombinant protein.

Recombinant expression vectors can be introduced into host cells to produce a transformed host cell. The term "transformed host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with",
10 "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate
15 or calcium chloride co-precipitation, DEAE-dextran mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic host
20 cells. For example, the proteins of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1991).

More particularly, bacterial host cells suitable for carrying out the present
25 invention include *E. coli*, *B. subtilis*, *Salmonella typhimurium*, and various species within the genus *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, as well as many other bacterial species well known to one of ordinary skill in the art. Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication.
30 Representative promoters include the β -lactamase (penicillinase) and lactose promoter system (see Chang et al., *Nature* 275:615 (1978)), the trp promoter (Nichols and Yanofsky, *Meth in Enzymology* 101:155, (1983) and the tac promoter (Russell et al., *Gene* 20:231, (1982)). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Suitable expression
35 vectors include but are not limited to bacteriophages such as lambda derivatives or plasmids such as pBR322 (Bolivar et al., *Gene* 2:95, (1977)), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, *Meth in Enzymology* 101:20-77, 1983 and Vieira

Summers, M.D., *Virology* 170:31-39 (1989)). Some baculovirus-insect cell expression systems suitable for expression of the recombinant proteins of the invention are described in PCT/US/02442.

Alternatively, the proteins of the invention may also be expressed in non-human transgenic animals such as, rats, rabbits, sheep and pigs (Hammer et al. *Nature* 315:680-683 (1985); Palmiter et al. *Science* 222:809-814 (1983); Brinster et al. *Proc. Natl. Acad. Sci. USA* 82:4438-4442 (1985); Palmiter and Brinster *Cell* 41:343-345 (1985) and U.S. Patent No. 4,736,866).

The proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, *J. Am. Chem. Assoc.* 85:2149-2154 (1964)) or synthesis in homogenous solution (Houbenweyl, *Methods of Organic Chemistry*, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart (1987)).

The present invention also provides proteins comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a specific protease. Such a protein could be prepared other than by recombinant means, for example by chemical synthesis or by conjugation of A and B chains and a linker sequence isolated and purified from their natural plant, fungal or bacterial source. Such A and B chains could be prepared having the glycosylation pattern of the native ricin-like toxin.

N-terminal or C-terminal fusion proteins comprising the protein of the invention conjugated with other molecules, such as proteins may be prepared by fusing, through recombinant techniques. The resultant fusion proteins contain a protein of the invention fused to the selected protein or marker protein as described herein. The recombinant protein of the invention may also be conjugated to other proteins by known techniques. For example, the proteins may be coupled using heterobifunctional thiol-containing linkers as described in WO 90/10457, N-succinimidyl-3-(2-pyridyldithio-propionate) or N-succinimidyl-5 thioacetate. Examples of proteins which may be used to prepare fusion proteins or conjugates include cell binding proteins such as immunoglobulins, hormones, growth factors, lectins, insulin, low density lipoprotein, glucagon, endorphins, transferrin, bombesin, asialoglycoprotein glutathione-S-transferase (GST), hemagglutinin (HA), and truncated myc.

III. Utility of the Nucleic Acid Molecules and Proteins of the Invention

(a) Therapeutic Methods

As mentioned above, matrix metalloproteinases (MMPs or matrixins) are zinc-dependent proteinases and the expression of MMP genes is reported to be activated in inflammatory disorders (e.g. rheumatoid arthritis) and malignancy. In addition, there

are reports of increased activation and expression of urokinase type plasminogen activator in inflammatory disorders such as rheumatoid arthritis (Slot, O., et al. 1999), osteoarthritis (Pap, G. et al., 2000), atherosclerotic cells (Falkenberg, M., et al., 1998) Crohn's disease (Desreumaux P, et al. 1999), central nervous system disease (Cuzner and
5 Opdenakker, 1999) as well as in malignancy. Accordingly, the recombinant proteins of the invention may be used to specifically inhibit or destroy cells that contain a specific protease that can cleave the linker sequence of the recombinant protein. More particularly, the recombinant proteins of the invention may be used to specifically inhibit or destroy cancer cells that contain a protease that can cleave the linker sequence
10 of the recombinant protein.

It is an advantage of the recombinant proteins of the invention that they have specificity for cells that contain a specific protease, including those of inflammatory disorders and cancer cells, without the need for a cell binding component. The ricin-like B chain of the recombinant proteins recognize galactose moieties on the
15 cell surface and ensure that the protein is taken up by, for example, a cancer cell and released into the cytoplasm. When the protein is internalized into a normal cell, cleavage of the heterologous linker would not occur in the absence of the specific protease, and the A chain will remain inactive bound to the B chain. Conversely, when the protein is internalized into a cell having a specific protease, the specific protease
20 will cleave the cleavage recognition site in the linker thereby releasing the toxic A chain.

Accordingly, the present invention provides a method of inhibiting or destroying cells having a specific protease, for examples inflammatory cells or cancer cells, comprising contacting such cells with an effective amount of a recombinant protein
25 or a nucleic acid molecule encoding a recombinant protein of the invention. The present invention also provides a method of treating a cell having a specific protease, comprising administering an effective amount of a recombinant protein or a nucleic acid molecule encoding a recombinant protein of the invention to an animal in need thereof.

The term "effective amount" as used herein means an amount effective, at
30 dosages and for periods of time necessary to achieve the desired result.

The term "animal" as used herein means any member of the animal kingdom including all mammals, birds, fish, reptiles and amphibians. Preferably, the animal to be treated is a mammal, more preferably a human.

The specificity of a recombinant protein of the invention may be tested by
35 treating the protein with the specific protease which is thought to be specific for the cleavage recognition site of the linker and assaying for cleavage products. For example, specific proteases may be isolated from cancer cells, or they may be prepared recombinantly, for example following the procedures in Darket et al. (*J. Biol. Chem.*

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254:2307-2312 (1988)). The cleavage products may be identified for example based on size, antigenicity or activity. The toxicity of the recombinant protein may be investigated by subjecting the cleavage products to an *in vitro* translation assay in cell lysates, for example using Brome Mosaic Virus mRNA as a template. Toxicity of the cleavage products may be determined using a ribosomal inactivation assay (Westby et al., *Bioconjugate Chem.* 3:377-382 (1992)). The effect of the cleavage products on protein synthesis may be measured in standardized assays of *in vitro* translation utilizing partially defined cell free systems composed for example of a reticulocyte lysate preparation as a source of ribosomes and various essential cofactors, such as mRNA template and amino acids. Use of radiolabelled amino acids in the mixture allows quantitation of incorporation of free amino acid precursors into trichloroacetic acid precipitable proteins. Rabbit reticulocyte lysates may be conveniently used (O'Hare, *FEBS Lett.* 273:200-204 (1990)).

The ability of the recombinant proteins of the invention to selectively inhibit or destroy cells having specific proteases may be readily tested *in vitro* using cell lines having the specific protease, such as cancer cell lines. The selective inhibitory effect of the recombinant proteins of the invention may be determined, for example, by demonstrating the selective inhibition of cellular proliferation in cancer cells or infected cells.

Toxicity may also be measured based on cell viability, for example, the viability of cancer and normal cell cultures exposed to the recombinant protein may be compared. Cell viability may be assessed by known techniques, such as trypan blue exclusion assays.

In another example, a number of models may be used to test the cytotoxicity of recombinant proteins having a heterologous linker sequence containing a cleavage recognition site for a cancer associated matrix metalloprotease. Thompson, E.W. et al. (*Breast Cancer Res. Treatment* 31:357-370 (1994)) has described a model for the determination of invasiveness of human breast cancer cells *in vitro* by measuring tumour cell-mediated proteolysis of extracellular matrix and tumour cell invasion of reconstituted basement membrane (collagen, laminin, fibronectin, Matrigel or gelatin). Other applicable cancer cell models include cultured ovarian adenocarcinoma cells (Young, T.N. et al. *Gynecol. Oncol.* 62:89-99 (1996); Moore, D.H. et al. *Gynecol. Oncol.* 65:78-82 (1997)), human follicular thyroid cancer cells (Demeure, M.J. et al., *World J. Surg.* 16:770-776 (1992)), human melanoma (A-2058) and fibrosarcoma (HT-1080) cell lines (Mackay, A.R. et al. *Lab. Invest.* 70:781 783 (1994)), and lung squamous (HS-24) and adenocarcinoma (SB-3) cell lines (Spiess, E. et al. *J. Histochem. Cytochem.* 42:917-929 (1994)). An *in vivo* test system involving the implantation of tumours and measurement of tumour growth and metastasis in athymic nude mice has also been described (Thompson,

E.W. et al., *Breast Cancer Res. Treatment* 31:357-370 (1994); Shi, Y.E. et al., *Cancer Res.* 53:1409-1415 (1993)).

Although the primary specificity of the proteins of the invention for cells having a specific protease is mediated by the specific cleavage of the cleavage recognition site of the linker, it will be appreciated that specific cell binding components may optionally be conjugated to the proteins of the invention. Such cell binding components may be expressed as fusion proteins with the proteins of the invention or the cell binding component may be physically or chemically coupled to the protein component. Examples of suitable cell binding components include antibodies to cancer proteins.

Antibodies having specificity for a cell surface protein may be prepared by conventional methods. A mammal, (e.g. a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g. the hybridoma technique originally developed by Kohler and Milstein (*Nature* 256:495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., *Immunol.Today* 4:72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., *Methods Enzymol*, 121:140-67 (1986)), and screening of combinatorial antibody libraries (Huse et al., *Science* 246:1275 (1989)). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated.

The term "antibody" as used herein is intended to include fragments thereof which also specifically react with a cell surface component. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, F(ab')₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human
5 constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes a cell surface antigen (See, for example, Morrison et al., *Proc. Natl Acad. Sci. U.S.A.* 81:6851 (1985); Takeda et al., *Nature* 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., E.P. Patent No. 171,496; European Patent No.
10 173,494; United Kingdom Patent No. GB 2177096B). It is expected that chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

Monoclonal or chimeric antibodies specifically reactive against cell surface components can be further humanized by producing human constant region chimeras, in
15 which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g. Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:7308-7312 (1983); Kozbor et al., *Immunology Today* 4:7279 (1983); Olsson et al., *Meth. Enzymol.*, 92:3-16 (1982), and PCT
20 Publication W092/06193 or EP 239,400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, reactive against cell surface components may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with cell surface
25 components. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., *Nature* 341:544-546 (1989); Huse et al., *Science* 246:1275-1281 (1989); and McCafferty et al., *Nature* 348:552-554 (1990)). Alternatively, a SCID-hu mouse, for example the model developed by Genpharm, can be used to produce antibodies, or fragments thereof.

30 **(b) Pharmaceutical Compositions**

The proteins and nucleic acids of the invention may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration in vivo" is meant a form of the substance to be administered in which any
35 toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary

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to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regime may be adjusted to provide the optimum therapeutic response. For
5 example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

Accordingly, the present invention provides a pharmaceutical composition for treating cells having a specific protease comprising a recombinant protein or a nucleic acid encoding a recombinant protein of the invention and a pharmaceutically acceptable
10 carrier, diluent or excipient.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, intramuscular, etc.), oral administration, inhalation, transdermal administration (such as topical cream or ointment, etc.), or suppository applications. Depending on the route of administration, the active substance
15 may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is
20 combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and
25 contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The pharmaceutical compositions may be used in methods for treating animals, including mammals, preferably humans, with cancer. It is anticipated that the compositions will be particularly useful for treating patients with B-cell
30 lymphoproliferative disease and melanoma. The dosage and type of recombinant protein to be administered will depend on a variety of factors which may be readily monitored in human subjects. Such factors include the etiology and severity (grade and stage) of the neoplasia.

The following non-limiting examples are illustrative of the present
35 invention:

EXAMPLES

EXAMPLE 1

Cloning and Expression of Proricin Variants Activated by Disease Specific Proteases

Isolation of total RNA

The preproricin gene was cloned from new foliage of the castor bean plant. Total messenger RNA was isolated according to established procedures (Sambrook et al., *Molecular Cloning: A Lab Manual* (Cold Spring Harbour Press, Cold Spring Harbour, 5 (1989)) and cDNA generated using reverse transcriptase.

cDNA Synthesis

Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene were synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., *Eur. J. Biochem.*, 145:266-270, 1985), several 10 oligonucleotide primers were designed to flank the start and stop codons of the preproricin open reading frame. The oligonucleotides were synthesized using an Applied Biosystems Model 392 DNA/RNA Synthesizer. First strand cDNA synthesis was primed using the oligonucleotide Ricin1729C. Three micrograms of total RNA was used as a template for oligo Ricin1729C (5'-ATAACTTGCTGCTCCTTTCA-3') primed synthesis of 15 cDNA using Superscript II Reverse Transcriptase (BRL) following the manufacturer's protocol.

DNA Amplification and Cloning

The first strand cDNA synthesis reaction was used as template for DNA amplification by the polymerase chain reaction (PCR). The preproricin cDNA was 20 amplified using the upstream primer Ricin-99 (5'-CCGGGAGGAAATACTATTGTAAT-3') and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). Amplification was carried out in a Biometra thermal cycler (TRIO-Thermalcycler) using the following 25 cycling parameters: denaturation 95°C for 1 min., annealing 52°C for 1 min., and extension 72°C for 2 min., (33 cycles), followed by a final extension cycle at 72°C for 10 min. The 1846 bp amplified product was fractionated on an agarose gel (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989), and the DNA purified from the gel slice using Qiaex resin (Qiagen) following the 30 manufacturer's protocol. The purified PCR fragment encoding the preproricin cDNA was then ligated (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)) into an Eco RV digested pBluescript II SK plasmid (Stratagene), and used to transform competent XL1-Blue cells (Stratagene). Positive clones were confirmed by restriction digestion of purified plasmid DNA. 35 Plasmid DNA was extracted using a Qiaprep Spin Plasmid Miniprep Kit (Qiagen).

DNA Sequencing

The cloned PCR product containing the putative preproricin gene (pAP144) was confirmed by DNA sequencing of the entire cDNA clone. Sequencing was performed

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using an Applied Biosystems 373A Automated DNA Sequencer, and confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (USB) (see WO 98/49311).

Production and Cloning of Linker Variants

5 pAP144 cut with EcoRI was used as target for PCR pairs employing the Ricin109-Eco oligonucleotide (Ricin-109Eco primer: 5-GGAGGAATCCGGAGATGAAACCGGGAGGAAATACTATTGTAAT-3) and a mutagenic primer for the 5' half of the linker as well as the Ricin1729PstI primer (Ricin 1729-PstI: 5-GTAGGCGCTGCAGATAACTTGCTGTCCTTTCAG-3) and a mutagenic primer for the 3' half of the linker. The cycling conditions used for the PCRs were 98 degrees C for 2 min.; 98°C 1 min., 52°C 1 min., 72°C 1 min. 15 sec. (30 cycles); 72 degrees C 10 min.; 4 degrees C soak. The PCR products were then digested by EcoRI and PstI respectively, electrophoresed on an agarose gel, and the bands purified by via glass wool spin columns: Triple ligations comprising the PCR product pairs (corresponding halves of the new linker) and pVL1393 vector digested with EcoRI and PstI were carried out. Recombinant clones were identified by restriction digests of plasmid miniprep DNA and the altered linkers confirmed by DNA sequencing. Note that all altered linker variants were cloned directly into the pVL1393 vector.

Isolation of Recombinant Baculoviruses

20 Insect cells *S. frugiperda* (Sf9), and *Trichoplusia ni* (Tn368 and BTI-TN-581-4 (High Five)) were maintained on EX-CELL 405 medium (JRH Biosciences) supplemented with 10% total calf serum (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Two micrograms of recombinant pVL1393 DNA was co-transfected with 0.5 microgram of 25 BaculoGold AcNPV DNA (Pharmingen) into 2×10^6 Tn368 insect cells following the manufacturer's protocol (Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)). On day 5 post-transfection, media were centrifuged and the supernatants tested in limiting dilution assays with Tn368 cells (Summers et al., A Manual of Methods of Baculovirus Vectors and 30 Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Recombinant viruses in the supernatants were then amplified by infecting Tn368 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 3 to 5 supernatants. A total of three rounds of amplification were performed for each recombinant following established procedures (Summers et al., A Manual of Methods of Baculovirus Vectors and 35 Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987 and Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)).

Expression of Mutant Proricin

Recombinant baculoviruses were used to infect 1×10^7 Tn368 or Sf9 cells at an moi of 9 in EX-CELL 405 media (JRH Biosciences) with 25mM α -lactose in spinner flasks. Media supernatants containing mutant proricins were collected 3 or 4 days post-infection.

EXAMPLE 2

5 Harvesting and affinity column purification of pro-ricin variants

Protein samples were harvested three days post infection. The cells were removed by centrifuging the media at 8288 g for ten minutes using a GS3 (Sorvall) centrifuge rotor. The supernatant was further clarified by centrifuging at 25400 g using a SLA-1500 rotor (Sorvall) for 45 minutes. Protease inhibitor phenylmethylsulfonyl fluoride (Sigma) was slowly added to a final concentration of 1 mM. The samples were further prepared by adding α -lactose to a concentration of 20 mM (not including the previous lactose contained in the expression medium). The samples were concentrated to 700 mL using a Prep/Scale-TFF Cartridge (2.5ft, 10K regenerated cellulose (Millipore)) and a Masterflex pump. The samples were then dialysed for 2 days in 1X Column Buffer (50 mM Tris, 100 mM NaCl, 0.02% NaN₃, pH 7.5) using dialysis tubing (10 K MWCO, 32 mm flat width(Spectra/Por)). Subsequently, the samples were clarified by centrifuging at 25400 g using a SLA-1500 rotor (Sorvall) for 45 minutes.

Following centrifugation, the samples were degassed and applied at 4 degrees C to a XK26/20 (Pharmacia) column (attached to a Pharmacia peristaltic pump, Pharmacia Single-path Monitor UV-1 Control and Optical Units, and Bromma LKB 2210 2-Channel Recorder) containing 20 mL α -Lactose Agarose Resin (Sigma). The column was washed for 3 hours with 1X Column buffer. Elution of proricin variant was performed by eluting with buffer (1X Column buffer (0.1% NaN₃), 100 mM Lactose) until the baseline was again restored. The samples were concentrated using an Amicon 8050 concentrator (Amicon) with a YM10 76 mm membrane, utilizing argon gas to pressurize the chamber. The samples were further concentrated in Centricon 10 (Millipore) concentrators according to manufacturer's specifications.

Purification of Variant PAP-Protein by gel filtration chromatography

In order to purify variant from processed material produced during fermentation, the protein was applied to a SUPERDEX 75 (16/60) column and SUPERDEX 200 (16/60) column (Pharmacia) connected in series equilibrated with 100 mM Tris, 200 mM NaCl, pH 7.5 containing 100 mM lactose and 1.0% β -mercaptoethanol (β ME). The flow rate of the column was 0.15 mL/min and fractions were collected every 25 minutes. The UV (280 nm) trace was used to determine the approximate location of the purified PAP-protein and thus determine the samples for Western analysis.

Western analysis of column fractions

Fractions eluted from the SUPERDEX columns (Pharmacia) were analyzed for purity using standard Western blotting techniques. An aliquot of 10 μ L from each fraction

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1:3000 in 1X PBS containing 0.5% ovalbumin and 0.1% Tween-20, added at 100 μ L per well and incubated for 1 h at 37°C. The plate was washed as above. Donkey anti- rabbit polyclonal antibody was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin, 0.1% Tween-20, and added at 100 μ L per well and incubated for 1 h at 37°C. The plate was
 5 given a final wash as described above. Substrate was added to plate at 100 μ L per well (1 mg/mL o-phenylenediamine (in H₂O), 1 μ L/mL H₂O₂) and after development 25 μ L of stop solution (20% H₂SO₄) was added and the absorbance read (A490nm-A630nm) using a SPECTRA MAX 340 plate reader (Molecular Devices).

Determination of PAP-Protein activity using the rabbit reticulocyte assay

10 Ricin samples were prepared for reduction.

A) RCA₆₀ = 3,500 ng/ μ L of RCA₆₀ + 997 μ L 1x Endo buffer (25 mM Tris, 25 mM KCl, 5mM MGC1₂, pH 7.6)

Reduction = 95 μ L of 10 ng/ μ L + 5 μ L β -mercaptoethanol

B) Ricin variants

15 Reduction = 40 μ L variant + 2 μ L β -mercaptoethanol

The ricin standard and the variants were incubated for 30 minutes at room temperature.

Ricin - Rabbit Reticulocyte lysate reaction

The required number of 0.5 mL tubes were labelled. (2 25 tubes for each
 20 sample, + and - aniline). To each of the sample tubes 20 μ L of 1X endo buffer was added, and 30 μ L of buffer was added to the controls. To the sample tubes either 10 μ L of 10ng/ μ L, Ricin or 10 μ L of variant was added. Finally, 30 μ L of rabbit reticulocyte lysate was added to all the tubes. The samples were incubated for 30 minutes at 30°C using the thermal block. Samples were removed from the 0.5 mL tube and contents added into a 1.5
 25 mL tube containing 1 mL of TRIZOL (Gibco). Samples were incubated for 15 minutes at room temperature. After the incubation, 200 μ L of chloroform was added, and the sample was vortexed and spun at 12,000 g for 15 minutes at 4°C. The top aqueous layer from the samples was removed and contents added to a 1 mL tube containing 500 μ L of isopropanol. Samples were incubated for 15 minutes at room temperature and then centrifuged at 12,000
 30 for 15 minutes at 4°C. Supernatant was removed and the pellets were washed with 1 mL of 70% ethanol. Centrifugation at 12,000 g for 5 minutes at 4°C pelleted the RNA. All but approximately 20 μ L of the supernatant was removed and the RNA pellet was allowed to air dry. Pellets from the other samples (+aniline samples) were dissolved in 20 μ L of DEPC treated ddH₂O. An 80 μ L aliquot of 1 M aniline (distilled) with 2.8 M acetic acid
 35 was added to these RNA samples and transferred to a fresh 0.5 mL tube. The samples were incubated in the dark for 3 minutes at 60°C. RNA was precipitated by adding 100 μ L of 95% ethanol and 5 μ L of 3M sodium acetate, pH 5.2 to each tube and centrifuging at

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12,000 g for 30 minutes at 4°C. Pellets were washed with 1 mL 70% ethanol and centrifuged again at 12,000g for 5 minutes at 4°C to precipitate RNA. The supernatant was removed and air dried. These pellets were dissolved in 10 µL of 0.1 X E buffer. To all samples, 10 µL of formamide loading dye was added. The RNA ladder (BRL) (8 µL of
5 ladder + 8 µL of loading dye) was also included. Samples were incubated for 2 minutes at 70°C on the thermal block. Electrophoresis was carried out on the samples using 1.2% agarose, 50% formamide gels in 0.1X E buffer + 0.2% SDS. The gel was run for 90 minutes at 75 volts. RNA was visualized by staining the gel in 1 µg/µL ethidium bromide in running buffer for 45 minutes. The gel was examined on a 302 nm UV box, photographed
10 using the gel documentation system and saved to a computer disk.

Results:

Protein Expression Yields

Aliquots were taken at each stop of the harvesting/ purification and tested. Yields of functional ricin variant were determined by ELISA. Typical results of an 3400
15 mL prep of infected *T. ni* cells are given below.

<u>Aliquot</u>	<u>µg PAP 304</u>
Before concentration and dialysis	14,472
after concentration and dialysis	13,611
alpha- Lactose agarose column flow through	418
20 alpha- Lactose agarose column elution	8,682

Yield: 8,682/14,472 = 60%

Purification of PAP-Protein and Western Analysis of column fractions

Partially purified PAP-protein was applied to Superdex 75 and 200
25 (16/60) columns connected in series in order to remove the contaminating non-specifically processed PAP-protein. Eluted fractions were tested via Western analysis as described above and the fractions containing the most pure protein were pooled, concentrated and dialyzed against 1 X PBS buffer and then sterilized by filtration (Millipore). Final purified PAP-protein has less than 1% processed variant.

30 The purified PAP-protein was tested for susceptibility to cleavage by the particular protease and for activation of the A chain of the proricin variant, (inhibition of protein synthesis). Typically, PAP-protein was incubated with and without protease for a specified time period and then electrophoresed and blotted. Cleaved PAP-protein will run as two 30 kDa proteins (B is slightly larger) under reducing
35 (SDS-PAGE) conditions. Unprocessed PAP-protein, which contains the linker region, will migrate at 60 kDa.

Activation of PAP -Protein variant with Specific Protease

Activation of protease treated PAP-protein is based on the method of May *et al.* (EMBO Journal. 8 301-8, 1989). Activation of ricin A chain upon cleavage of the intermediary linker results in catalytic depurination of the adenosine 4325 residue of 28S or 26S rRNA. This depurination renders the molecule susceptible to amine-catalyzed hydrolysis by aniline of the phosphodiester bond on either side of the modification site. The result is a diagnostic 390 base band. As such, reticulocyte ribosomes incubated with biochemically purified ricin A chain, released the characteristic RNA fragment upon aniline treatment of isolated rRNA (May, M.J. *et al.* Embo. Journal, 8:301-308 at 302- 303 (1989)). It is on this basis that the assay allows for the determination of activity of a ricin A chain which has been cleaved from the intact unit containing a particular variant linker sequence.

EXAMPLE 3

In Vitro Protease Digestion of Proricin Variants:

Affinity-purified proricin variant is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. Ricin-like toxin variants are eluted from the lactose-agarose matrix in protease digestion buffer (50mM NaCl, 50mM Na-acetate, pH 5.5, 1mM dithiothreitol) containing 100mM lactose. Proricin substrate is then incubated at 37°C for 60 minutes with a disease-specific protease. The cleavage products consisting ricin A and B chains are identified using SDS/PAGE (Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma). Figure 19 shows the cleavage products of an MMP-9 digestion of PAP323, PAP324 and PAP325.

Matrix metalloproteinases may be prepared substantially as described by Lark, M.W. *et al.* (*Proceedings of the 4th International Conference of the Inflammation Research Association* Abstract 145 (1988)) and Welch, A.R. *et al.* (Arch. Biochem. Biophys. 324:59-64 (1995)).

Urokinase plasminogen activator may be prepared substantially as described by Holmberg, L. *et al.* (Biochim Biophys Acta, 445:215-222, (1976)) and Someno, T. *et al.* (J Biochem 97:1493-1500 (1985)).

EXAMPLE 4

Cytotoxicity of Ricin and Ricin Variants on Cell Lines

Cell Lines

COS-1 (African Green Monkey Kidney Cells)

This is an SV40 transformed cell line which was prepared from established simian cells CV-1. (Reference: Gluzman, Y. (1975) Cell, 23, 175 - 182)(ATCC CRL 1650).

HT-1080 Human Fibrosarcoma

(ATCC CCL 121) This cell line was shown to produce active MMP-9 in tissue culture. (References: Moore et al. (1997) Gynecologic Oncology 65, 83-88.)

Cell Preparation

After washing with 1XPBS (0.137 M NaCl, 2.68 mM KCl, 8.10 mM Na₂HPO₄, 1.47 mM KH₂PO₄), cells in log phase growth were removed from plates with 1X trypsin/EDTA (Gibco/BRL). The cells were centrifuged at 1100 rpm for 3 min, resuspended in Dulbecco's Modified Eagle Medium containing 10%FBS and 1X pen/strep, and then counted using a haemocytometer. They were adjusted to a concentration of 5 X 10⁴ cells•ml⁻¹. One hundred microliters per well of cells was added to wells 2B - 2G through to wells 9B - 9G of a Falcon 96 well tissue culture plate. A separate 96 well tissue culture plate was used for each sample of Ricin or Ricin variant. The plates were incubated at 37°C with 5% CO₂ for 24 hours.

Toxin Preparation

The Ricin and Ricin variants were sterile filtered using a 0.22 µm filter (Millipore). The concentration of the sterile samples were then quantified by A₂₈₀ and confirmed by BCA measurements (Pierce). For the variants digested with the MMP-9 protease in vitro, the digests were carried out as described in the digestion procedure for each protease. The digests were then diluted in the 1000 ng•ml⁻¹ dilution and sterile filtered. Ricin and Ricin variants were serially diluted to the following concentrations: 1000 ng•ml⁻¹, 100 ng•ml⁻¹, 10 ng•ml⁻¹, 1 ng•ml⁻¹, 0.1 ng•ml⁻¹, 0.01 ng•ml⁻¹, 0.001 ng•ml⁻¹ with media containing 10%FBS and 1X pen/strep.

Application of Toxin or Variants to Plates

Columns 2 to 9 were labeled: control, 1000 ng•ml⁻¹, 100 ng•ml⁻¹, 10 ng•ml⁻¹, 1 ng•ml⁻¹, 0.1 ng•ml⁻¹, 0.01 ng•ml⁻¹, 0.001 ng•ml⁻¹ consecutively. The media was removed from all the sample wells with a multichannel pipettor. For each plate of variant and toxin, 50 µl of media was added to wells 2B to 2G as the control, and 50 µl of each sample dilution was added to the corresponding columns. The plates were incubated for one hour at 37°C with 5% CO₂, then washed once and replaced with media, then incubated for 48 hours at 37°C with 5% CO₂.

Sample Application

The whole amount of media (and/or toxin) was removed from each well with a multichannel pipettor, and replaced with 100 µl of the substrate mixture (Promega Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit). The plates were incubated at 37°C with 5% CO₂ for 2 to 4 hours, and subsequently read with a Spectramax 340 96 well plate reader at 490nm. The IC₅₀ values were calculated using the GRAFIT software program.

Results

Subcutaneous A431 tumours were established in SCID mice. The tumours were treated with either PAP304 or PAP305 when the tumours reached 50 mm³ on Days 1, 5 and 9. The results shown in Figures 20 and 21 demonstrate that the linker decreases the toxicity of the variant (as compared with ricin) and the variants PAP304 and PAP305 are activated at or near the A431 (human epithelial carcinoma) solid tumour in mice. A very exciting result is shown in Figure 20. In this study, the variant PAP304 was able to slow down the growth of A431 solid tumour (17 day delay), without any signs of dose limiting toxicity (e.g., no weight loss or death).

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

FULL CITATIONS FOR CERTAIN REFERENCES REFERRED TO IN THE SPECIFICATION

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1) Cytotoxicity of Selected Variants

Table 1: Selected Variants against COS-1 Cells – Target Protease MMP-9

	Ricin	PAP220	PAP301	PAP302	PAP303	PAP304	PAP305	PAP308
Linker Length (residues)	-	23	23	16	15	8	12	12
Reduction in toxicity relative to Ricin	1X	23X	24X	118X	63X	1220X	145X	89X

Table 2: Selected Variants against HT1080 Cells – Target Protease MMP-9

	Ricin	PAP220	PAP301	PAP302	PAP303	PAP304	PAP305	PAP308
Linker Length (residues)	-	23	23	16	15	8	12	12
Reduction in toxicity relative to Ricin	1X	4X	5X	24X	12X	137X	38X	21X

2) Cytotoxicity Data from Selected Variants

Table 3: Selected Variants against COS-1 cells

MMP9 Variants

	Ricin	PAP316	PAP318	PAP323	PAP324	PAP325
Linker Length (residues)	-	23	23	21	19	17
Reduction in toxicity relative to Ricin	1X	39X	100X	65X	67X	82X

UPA Variants

	Ricin	PAP313	PAP314	PAP315	PAP320	PAP321	PAP322
Linker Length (residues)	-	7	15	14	13	11	9
Reduction in toxicity relative to Ricin	1X	110X	52X	75X	55X	1283X	82X

Table 4: Selected Variants against HT1080 Cells

MMP9 Variants

	Ricin	PAP316	PAP318	PAP323	PAP324	PAP325
Linker Length (residues)	-	23	23	21	19	17
Reduction in toxicity relative to Ricin	1X	13X	51X	15X	14X	20X

UPA Variants

	Ricin	PAP313	PAP314	PAP315	PAP320	PAP321	PAP322
Linker Length (residues)	-	7	15	14	13	11	9
Reduction in toxicity relative to Ricin	1X	43X	27X	18X	14X	367X	51X

Table 5: Maximum Tolerable Dose of MMP9 Variants

MMP9 Variant	Linker Size	<i>In Vivo</i> (µg/kg)
PAP301	23	8
PAP302	16	40
PAP303	15	10
PAP304	8	150
PAP305	12	20
PAP308	12	30
PAP309	23	20
PAP316	23	20
PAP318	23	<20
PAP323	21	15
PAP324	19	20
PAP325	17	20

(cf. Ricin - 1.6 µg/kg and PAP220 - 13 µg/kg)

Group	Sample	Drug Dose (µg/kg)	Treatment (days)
1	Control – Buffer	0	1, 5, and 9
2	PAP304	75	1, 5, and 9
3	PAP304	100	1, 5, and 9
4	PAP304	150	1, 5, and 9

WE CLAIM:

1. A purified and isolated nucleic acid molecule comprising (a) a nucleotide sequence encoding an A chain of a ricin-like toxin, (b) a nucleotide sequence encoding a B chain of a ricin-like toxin and (c) a nucleotide sequence encoding a heterologous linker amino acid sequence linking the A and B chains, the heterologous linker sequence containing a cleavage recognition site for a specific protease.
5
2. A nucleic acid molecule of claim 1 wherein the specific protease is an MMP or UPA.
3. A nucleic acid molecule according to claim 1 or 2 wherein the protease is associated with a cancer cell.
10
4. A nucleic acid molecule according to claim 3 wherein the cancer cell is one found in T- and B-cell lymphoproliferative diseases, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate, cancer or non small cell lung cancer.
- 15 5. A nucleic acid molecule according to claim 1 or 2 wherein the protease is associated with an inflammatory cell.
6. A nucleic acid molecule according to claim 5 wherein the cell is one found in rheumatoid arthritis, atherosclerotic cells, Crohn's disease, or central nervous system disease.
- 20 7. A nucleic acid molecule of anyone of claims 1-6 wherein the A chain is ricin A chain, abrin toxin A chain, diphtheria toxin A chain, Domain III of Pseudomonas exotoxin, volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain, viscumin toxin A chain or shiga toxin A chain.
8. A nucleic acid molecule of any one of claims 1-6 wherein the B chain is ricin B chain, abrin toxin B chain, diphtheria toxin B chain, Domain I/II of Pseudomonas exotoxin, volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain, viscumin toxin B chain or shiga toxin B chain.
25
9. A nucleic acid molecule according to any one of claims 1 to 8 having a nucleic acid sequence selected from the group consisting of the nucleic acid sequence of pAP301 as

shown in Figure 1B; the nucleic acid sequence of pAP302 as shown in Figure 2B; the nucleic acid sequence of pAP303 as shown in Figure 3B; the nucleic acid sequence of pAP304 as shown in Figure 4B; the nucleic acid sequence of pAP305 as shown in Figure 5B; the nucleic acid sequence of pAP308 as shown in Figure 6B; the nucleic acid sequence of pAP309 as shown in Figure 7B; the nucleic acid sequence of pAP313 as shown in Figure 8B; the nucleic acid sequence of pAP314 as shown in Figure 9B; the nucleic acid sequence of pAP315 as shown in Figure 10B; the nucleic acid sequence of pAP316 as shown in Figure 11B; the nucleic acid sequence of pAP318 as shown in Figure 12B; the nucleic acid sequence of pAP320 as shown in Figure 13B; the nucleic acid sequence of pAP321 as shown in Figure 14B; the nucleic acid sequence of pAP322 as shown in Figure 15B; the nucleic acid sequence of pAP323 as shown in Figure 16B; the nucleic acid sequence of pAP324 as shown in Figure 17B; and the nucleic acid sequence of pAP325 as shown in Figure 18B.

10. A nucleic acid molecule according to any one of claims 1 to 8 wherein the nucleotide sequence of the linker is selected from the group consisting of: the nucleic acid sequence of pAP301 as shown in Figure 1A; the nucleic acid sequence of pAP302 as shown in Figure 2A; the nucleic acid sequence of pAP303 as shown in Figure 3A; the nucleic acid sequence of pAP304 as shown in Figure 4A; the nucleic acid sequence of pAP305 as shown in Figure 5A; the nucleic acid sequence of pAP308 as shown in Figure 6A; the nucleic acid sequence of pAP309 as shown in Figure 7A; the nucleic acid sequence of pAP313 as shown in Figure 8A; the nucleic acid sequence of pAP314 as shown in Figure 9A; the nucleic acid sequence of pAP315 as shown in Figure 10A; the nucleic acid sequence of pAP316 as shown in Figure 11A; the nucleic acid sequence of pAP318 as shown in Figure 12A; the nucleic acid sequence of pAP320 as shown in Figure 13A; the nucleic acid sequence of pAP321 as shown in Figure 14A; the nucleic acid sequence of pAP322 as shown in Figure 15A; the nucleic acid sequence of pAP323 as shown in Figure 16A; the nucleic acid sequence of pAP324 as shown in Figure 17A; and the nucleic acid sequence of pAP325 as shown in Figure 18A.

11. A plasmid incorporating the nucleic acid molecule of any one of claims 1 to 10.

12. A baculovirus transfer vector incorporating the nucleic acid molecule according to any one of claims 1 to 10.

13. A recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a specific protease.

14. A recombinant protein of claim 13 wherein the specific protease is an MMP or UPA.
15. A protein according to claim 13 or 14 wherein the protease is associated with a cancer cell.
16. A protein according to claim 15 wherein the cancer cell is one found in T- and B-cell lymphoproliferative diseases, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate, cancer or non small cell lung cancer.
17. A protein according to claim 13 or 14 wherein the protease is associated with an inflammatory cell.
18. A protein according to claim 17 wherein the cell is one found in rheumatoid arthritis, atherosclerotic cells, Crohn's disease, or central nervous system disease.
19. A recombinant protein of anyone of claims 13-18 wherein the A chain is ricin A chain, abrin toxin A chain, diphtheria toxin A chain, Domain III of Pseudomonas exotoxin, volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain, viscumin toxin A chain, or shiga toxin A chain.
20. A recombinant protein of anyone of claims 13-18 wherein the B chain is ricin B chain, abrin toxin B chain, diphtheria toxin B chain, Domain I/II of Pseudomonas exotoxin, volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain, viscumin toxin B chain, or shiga toxin B chain.
21. A recombinant protein of claim 13 wherein the linker amino acid sequence is selected from the group consisting of: the amino acid sequence of PAP301 as shown in Figure 1C; the amino acid sequence of PAP302 as shown in Figure 2C; the amino acid sequence of PAP303 as shown in Figure 3C; the amino acid sequence of PAP304 as shown in Figure 4C; the amino acid sequence of PAP305 as shown in Figure 5C; the amino acid sequence of PAP308 as shown in Figure 6C; the amino acid sequence of PAP309 as shown in Figure 7C; the amino acid sequence of PAP313 as shown in Figure 8C; the amino acid sequence of PAP314 as shown in Figure 9C; the amino acid sequence of PAP315 as shown in Figure 10C; the amino acid sequence of PAP316 as shown in Figure 11C; the amino acid sequence of PAP318 as shown in Figure 12C; the amino acid sequence of PAP320 as shown

in Figure 13C; the amino acid sequence of PAP321 as shown in Figure 14C; the amino acid sequence of PAP322 as shown in Figure 15C; the amino acid sequence of PAP323 as shown in Figure 16C; the amino acid sequence of PAP324 as shown in Figure 17C; and the amino acid sequence of PAP325 as shown in Figure 18C.

- 5 22. A method of inhibiting or destroying cells having a specific protease comprising the steps of:
- (a) preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin, and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker
- 10 sequence contains a cleavage recognition site for the protease;
- (b) introducing the nucleic acid into a host cell and expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a linker amino acid sequence;
- (c) suspending the protein in a pharmaceutically acceptable carrier, diluent or
- 15 excipient, and
- (d) contacting the cells with the recombinant protein.
23. A method according to claim 22 wherein the protease is an MMP or UPA.
24. A method according to claim 22 or 23 wherein the protease is associated with a cancer cell.
- 20 25. A method according to claim 24 wherein the cancer cell is one found in T- and B-cell lymphoproliferative diseases, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate, cancer or non small cell lung cancer.
26. A method according to claim 22 or 23 wherein the protease is associated with an
- 25 inflammatory cell.
27. A method according to claim 26 wherein the cell is one found in rheumatoid arthritis, atherosclerotic cells, Crohn's disease, or central nervous system disease.
28. A method of inhibiting or destroying cells having a specific protease comprising contacting the cells with an effective amount a recombinant protein according to any one
- 30 of claims 13 to 21.

29. A method of treating a cell having a specific protease comprising administering an effective amount of a recombinant protein according to any one of claims 13 to 21 to an animal in need thereof.
30. A method of treating a cell having a specific protease comprising administering
5 an effective amount of a nucleic acid molecule according to any one of claims 1 to 10 to an animal in need thereof.
31. A process for preparing a pharmaceutical for treating a cell having a specific protease comprising the steps of :
- 10 (a) preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin, and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a specific protease;
- (b) introducing the nucleic acid into a host cell and expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a
15 B chain of a ricin-like toxin and a linker amino acid sequence;
- (c) suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.
32. A process according to claim 31 wherein the protease is an MMP or UPA.
33. A process according to claim 31 or 32 wherein the protease is associated with a
20 cancer cell.
34. A process according to claim 33 wherein the cancer cell is one found in T- and B-cell lymphoproliferative diseases, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate, cancer or non small cell lung cancer.
35. A process according to claim 31 or 32 wherein the protease is associated with an
25 inflammatory cell.
36. A process according to claim 35 wherein the cell is one found in rheumatoid arthritis, atherosclerotic cells, Crohn's disease, or central nervous system disease.

37. A pharmaceutical composition for treating cancer comprising a recombinant protein of any one of claims 13 to 21 and a pharmaceutically acceptable carrier, diluent or excipient.
38. A pharmaceutical composition for treating inflammation comprising a
5 recombinant protein of any one of claims 13 to 21 and a pharmaceutically acceptable carrier, diluent or excipient.
39. A pharmaceutical composition for treating a cell having a specific protease comprising a nucleic acid molecule of any one of claims 1 to 10 and a pharmaceutically acceptable carrier, diluent or excipient.
- 10 40. A pharmaceutical composition for treating a cell having a specific protease comprising an amino acid molecule of any one of claims 1 to 10 and a pharmaceutically acceptable carrier, diluent or excipient.
41. A purified and isolated nucleic acid molecule having a nucleic acid sequence selected from the group consisting of: the nucleic acid sequence of pAP301 as shown in
15 Figure 1A; the nucleic acid sequence of pAP302 as shown in Figure 2A; the nucleic acid sequence of pAP303 as shown in Figure 3A; the nucleic acid sequence of pAP304 as shown in Figure 4A; the nucleic acid sequence of pAP305 as shown in Figure 5A; the nucleic acid sequence of pAP308 as shown in Figure 6A; the nucleic acid sequence of pAP309 as shown in Figure 7A; the nucleic acid sequence of pAP313 as shown in Figure 8A; the nucleic acid
20 sequence of pAP314 as shown in Figure 9A; the nucleic acid sequence of pAP315 as shown in Figure 10A; the nucleic acid sequence of pAP316 as shown in Figure 11A; the nucleic acid sequence of pAP318 as shown in Figure 12A; the nucleic acid sequence of pAP320 as shown in Figure 13A; the nucleic acid sequence of pAP321 as shown in Figure 14A; the nucleic acid sequence of pAP322 as shown in Figure 15A; the nucleic acid sequence of pAP323 as
25 shown in Figure 16A; the nucleic acid sequence of pAP324 as shown in Figure 17A; and the nucleic acid sequence of pAP325 as shown in Figure 18A.
42. A linker protein having an amino acid sequence selected from the group consisting of: the amino acid sequence of PAP301 as shown in Figure 1C; the amino acid sequence of PAP302 as shown in Figure 2C; the amino acid sequence of PAP303 as shown in
30 Figure 3C; the amino acid sequence of PAP304 as shown in Figure 4C; the amino acid sequence of PAP305 as shown in Figure 5C; the amino acid sequence of PAP308 as shown in Figure 6C; the amino acid sequence of PAP309 as shown in Figure 7C; the amino acid sequence of PAP313 as shown in Figure 8C; the amino acid sequence of PAP314 as shown in

Figure 9C; the amino acid sequence of PAP315 as shown in Figure 10C; the amino acid sequence of PAP316 as shown in Figure 11C; the amino acid sequence of PAP318 as shown in Figure 12C; the amino acid sequence of PAP320 as shown in Figure 13C; the amino acid sequence of PAP321 as shown in Figure 14C; the amino acid sequence of PAP322 as shown in Figure 15C; the amino acid sequence of PAP323 as shown in Figure 16C; the amino acid sequence of PAP324 as shown in Figure 17C; and the amino acid sequence of PAP325 as shown in Figure 18C.

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(54) Title: IMPROVED RICIN-LIKE TOXINS FOR TREATMENT OF CANCER

(57) Abstract: The present invention provides a protein having chain of a ricin-like toxin, a B chain of a ricin-like toxin and a novel heterologous linker amino acid sequence, linking the A and B chains. The linker sequence contains a cleavage recognition site for a specific protease such as those found in inflammatory cells and cancer cells. The invention also relates to a nucleic acid molecule encoding the protein and to expression vectors incorporating the nucleic acid molecule. Also provided is a method of inhibiting or destroying cells having a specific protease, such as cancer cells or inflammatory cells utilizing the nucleic acid molecules and proteins of the invention and pharmaceutical compositions for treating human inflammation and cancer.

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FIGURE 1A**Sequence of pAP301 (MMP-9) Linker Region****WT preprorincin linker**

primer 301-3'
 5' - ATGTGGGGACACGAAATTTTAATGCTGAT -3'
 * *** *
 -CTCATGGTGATAGATGGCGACCTCCACCATCGTCAAGTTTCTTTGCTTATA | AGGCCAGTGGTACCAAAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACGCGTGGAGGTGGTAGCAGTGTCAAAGAAACGAATAT | TCCGGTCACCATGGTTTAAATACGACTACAAACATACCTAGGACTCGGG-
 ** *** ***
 3' - GGTGGTAGCAGTGTCAAACCCAGGAGAACCG -5'
 primer 301-5'

1) PCR mutagenesis

2) Ligate with pVL1393

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pAP301 linker
 (MMP-9 variant)
 GCACCTCCACCATCGTCAAGTTTGGTCTCTTGGC | ATGTGGGACACGAAATTTTAATGCTGATGTT
 CGTGGAGGTGGTAGCAGTGTCAAACCCAGGAGAACCG | TACACCCCTGTTGCTTTAAATACGACTACAA

Note: Nucleotides in bold are found within the preprorincin linker region. The '-' symbol within the linker designate deleted nucleotides.

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FIGURE 1B (P1)

Sequence of pAP301 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTTCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGCG				
	CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGA CTACAAGTTTTA				
451	CGATATACATTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGA ACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCA ACT				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT C				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTAAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGGTCCTCTTGGCATGTGGGGACAACGAAATTTTAATGC
AGCAGTGTCAAACCAGGAGAACCGTACACCCCTGTTGCTTTAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTGAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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FIGURE 1B (P3)

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1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
    TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
    GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
    ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
    AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
    CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
    ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
    GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
    CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
    ACGTC
  
```

Total number of bases is: 1855.

Sequence name: pAP301

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

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FIGURE 1C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP301 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP301 (MMP-9) linker:	A chain- C A P P P S S Q F G P L G M W G Q R N F N A D V C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

Sequence of pAP302 (MMP-9) Linker Region

WT preproricin linker

primer 302-3' 5'- GGGCAG-----TGATGGATCCTGAGCCC -3'
 * **
 -CTCATGGTGTATAGATGCGCACCTCCACCATCGTCACAGTTTCTTTGCTTATAGGCCA | GTGGTACCAAAATTTTAAATGTGATGTTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACGCGTGGAGTGGTAGCAGTGTCAAAAGAAACGAAATATCCGGT | CACCATGGTTTAAAAATTACGACTACAAACATACCTAGGACTCGGG-
 ** **** ***
 3'- AGCAGTGTCAAAAGAGGCGTTTCCTTAACGT -5'
 primer 302-5'

- 1) PCR mutagenesis
- 2) Ligate with pVL1393

pAP302 linker
(MMP-9 variant)

GCACCTCCACCATCGTCACAGTTTCTCCGCAAGGAATTGCA	GGGCAG
CGTGGAGGTGGTAGCAGTGTCAAAGAGGGCGTTCTCTTAACGT	CCCGTC

Note: Nucleotides in bold are found within the preprorin linker region. The ‘.’ symbol within the linker designate deleted nucleotides.

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FIGURE 2B (P1)

Sequence of pAP302 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTGCTTC				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGCGCT				

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FIGURE 2B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTTCTCCGCAAGGAATTGCAGGGCAG-----
AGCAGTGTCAAAGAGGCGTTCCTTAAACGTCCCGTC-----

951 -----TGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
-----ACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTGTTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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FIGURE 2B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1834.

Sequence name: pAP302

Note: Nucleotides in bold are found within the mutant prepronicin linker region. The '-' symbol within the linker region designate deleted nucleotides.

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FIGURE 2C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP302 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP302 (MMP-9) linker:	A chain- C A P P S S Q F S P Q G I A G Q - - - - C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

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FIGURE 3A

Sequence of pAP303 (MMP-9) Linker Region

WT preprorin linker

primer 303-3'
 5'- GGGCAGCGAAATTTTAATGCTGAT -3'
 * *** *
 -CTCATGGTGTATAGATGGCACCTCCACCATCGTCACAGTTTCTTCTTATAAGGCCA|GTGGTACCAATTTTAATGCTGATGTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACGGGTGGAGGTGTAGCAGTGTCAAAAGAAACGAATATTCCGGT|CACCATGGTTTAAATACGACTACAAACATACCTAGGACTCGGG-
 ** **** ***
 3' -GAGTACCACATATCTACG-----AGAGGCGTTCCTTAACGT -5'
 primer 303-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP303 linker
 (MMP-9 variant)
 TCTCCGCAAGGAATTGCA|GGCAGCGAAATTTTAATGCTGATGTT
 AGAGGCGTTCCTTAACGT|CCGTCGCTTAAATACGACTACAA

Note: Nucleotides in bold are found within the preprorin linker region. The '-' symbol within the linker designate deleted nucleotides.

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FIGURE 3B (P1)

Sequence of pAP303 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACTGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTAGTTGAACTCTCA				
	TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAATAAGTTTGA				
451	CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAT				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTTC				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

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FIGURE 3B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGC-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACG-----

901 -----TCTCCGCAAGGAATTGCAGGGCAGCGAAATTTTAATGC
-----AGAGGCGTTCCTTAACGTCCCGTCGCTTTAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATCTCCTGACATCGTCACTTT

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FIGURE 3B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1831.

Sequence name: pAP303

Note: Nucleotides in bold are found within the mutant preprorcin linker region. The '-' symbol within the linker region designate deleted nucleotides.

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FIGURE 3C**Amino acid sequence Comparison of Mutant Preprorin Linker Region of PAP303 (MMP-9) to Wild Type**

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP303 (MMP-9) linker:	A chain- C - - - - - S P Q G I A G Q R N F N A D V C M D P E -B chain

Note: Amino acids in bold are found within the preprorin linker region. The '-' symbol within the linker designate deleted amino acids.

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FIGURE 4A**Sequence of pAP304 (MMP-9) Linker Region****WT preprorcin linker**

```

                    primer 304-3'
                    -----TGTATGGATCCTGAGCCC -3'
5'- GGGCAG-----
* ***
-CTCATGGTGATAGATGCGCACCTCCACCATCGTCACAGTTTCTTTGCTTATAAGGCCA|GTGGTACCAAAATTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
-GAGTACCACATATCTACGCGTGGAGGTGGTAGCAGTGTCAAAAGAAACGAATATTCCGGT|CACCATGGTTTAAATACGACTACAAACATACCTAGGACTCGGG-
** **** ***
3' -GAGTACCACATATCTACG-----AGAGGCGTTCCTTAACGT -5'
primer 304-5'

```

1) PCR mutagenesis

2) Ligase with pVL1393

```

pAP304 linker
(MMP-9 variant)
TCTCCGCAAGGAATTGCA|GGGCAG
AGAGGCGTTCCTTAACGT|CCCGTC

```

Note: Nucleotides in bold are found within the preprorcin linker region. The '.' symbol within the linker designate deleted nucleotides.

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FIGURE 4B (P1)

Sequence of pAP304 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGCG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTT				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG				
501	TGGTAATCTGAGAGAAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

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FIGURE 4B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGC-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACG-----

901 -----TCTCCGCAAGGAATTGCAGGGCAG-----
-----AGAGGCGTTCCTTAACGTCCCGTC-----

951 -----TGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCGAAATG
-----ACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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FIGURE 4B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1810.

Sequence name: pAP304

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

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FIGURE 4C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP304 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP304 (MMP-9) linker:	A chain- C - - - - S P Q G I A G Q - - - - C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

Sequence of pAP305 (MMP-9) Linker Region

WT preproricin linker

5' - GGGCAG - - - - - TGTATGGATCCTGAGCCC - 3',
 primer 305-3',
 + ***
 -CTCATGGTGTATAGATGGGCACCTCCACCATCGTCAAGTTTCTTTGCTTATAAGGCCA | GTGGTACCAAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC -
 -GAGTACCACATATCTACGCGTGGAGGTGTAGCAGTGTCAAAGAAACGAATATTCGGT | CACCATGGTTTAAATTTACGACTACAAACATACCTAGGACTCGGG -
 3' - TCTACGCGTGGAGGTGGT - - - - - AGAGGCGTTCCCTTAACGT - 5',
 primer 305-5',

- 1) PCR mutagenesis
- 2) Ligate with pVL1393

pAP 305 linker
(MMP-9 variant)

GCACCTCCACCATCTCCGCAAGGAATTGCA	GGGCAG
CGTGGAGGTGGTAGAGCGTTCCCTTAACGT	CCCGTC

Note: Nucleotides in bold are found within the preprorin linker region. The ‘.’ symbol within the linker designate deleted nucleotides.

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FIGURE 5B (P1)

Sequence of pAP305 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAACT				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGCG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACTGAGCTGATGTGAGACATGAAATACCAGTGTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAATAAAGTTTGA				
451	CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTG				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

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FIGURE 5B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 -----TCTCCGCAAGGAATTGCAGGGCAG-----
-----AGAGGCGTTCCTTAACGTCCCGTC-----

951 -----TGTATGGATCCTGAGCCCATAGTGCATATCGTAGGTCGAAATG
-----ACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 5C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP305 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P P S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP305 (MMP-9) linker:	A chain- C A P P P - - - S P Q G I A G Q - - - - C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

Sequence of pAP308 (MMP-9) Linker Region

WT preprorcin linker

5' - ATGTGGGACAA-----TGTGTTGGCGAGGGCCCATAGTGGTATCGTA -3
 primer 308-3'
 * * * * *
 *** * * * *
 -CTCATGGTGTATAGATGCGGCACCTCCACCATCGTCACAGTTTCTTTGCTTATA | AGCCAGTGGTACCAAAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCCATAGTGGGTATCGTA-
 -GAGTACCACATATCTACGGCGTGGAGGTGGTAGCAGTGTCAAAAAGAAACGAAATAT | TCCGGTCACCATGGTTTTAAATTACGACTACAAACATACCTAGGACTCGGGTATCAGCGCATAGCAT-
 * * * * *
 ** * * *
 3' - TCTACGGCTGGAGGTGGT-----CCAGGAGAACCG -5'
 primer 308-5'

- 1) PCR mutagenesis
- 2) Ligate with pVL1393

pAP 308 linker
(MMP-9 variant)
GCACCTCCACCAAGTCTCTTGGC | ATGTGGGACAA
CGTGGAGTGGTCCAGAGAACCG | TACACCCCTGTT

Note: Nucleotides in bold are found within the preprorin linker region. The ‘-’ symbol within the linker designate deleted nucleotides.

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FIGURE 6B (P1)

Sequence of pAP308 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGCG				
	CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTACGCGTGCTCTTAATCCATGTTGGCCT				

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FIGURE 6B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 -----GGTCCTCTTGGCATGTGGGGACAA-----
-----CCAGGAGAACCGTACACCCCTGTT-----

951 -----TGTGGTGGCGGAGGGCCCATAGTGCGTATCGTAGGTCGAAATG
-----ACACCACCGCCTCCCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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FIGURE 6B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCACTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1822.

Sequence name: pAP308

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 6C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP308 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E - B chain
PAP308 (MMP-9) linker:	A chain- C A P P P - - - - G P L G M W G Q - - - - - C G G G G - B chain

Note: Amino acids in bold are found within the preproricin linker region. The ‘.’ symbol within the linker designate deleted amino acids.

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FIGURE 7A

Sequence of pAP309 (MMP-9) Linker Region

WT preporicilin linker

```

                    primer 309-3'
5'- TTTAATGCTGATGTTTGTGTGGCGGAGGCCCATAGTCGTATCGTA -3
    *** ***** *
IGGTGTATAGATGCGCACCTCCACCATCGTCACAGTTTCTTTGCTTATAAGCCAGTGGTACCAAAT | TTTAATGCTGATGTTTGTATGGATCCTGAGCCCATAGTCGTATCGTA-
ACCACATATCTACGCGTGGAGTGGTAGCAGTGTCAAAGAAACGAATATTCGGTCAACCATGGTTTA | AAATTACGACTACAAACATACCTAGGACTCGGGGTATCAGGCATAGCAT-
    ** *** *** ***** *
3'- GGTGGTAGCAGTGTCAAACCCAGGAGAACCGTACACCCCTGTTGCTTTA -5'
    primer 309-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

```

pAP309 linker
(MMP-9 variant)
GCACCTCCACCATCGTCACAGTTTGGTCTCTTGGCATGTGGGACAAACGAAAT | TTTAATGCTGATGTT
CGTGGAGTGGTAGCAGTGTCAAACGAGAGAACCGTACACCCCTGTTGCTTTA | AAATTACGACTACAA

```

Note: Nucleotides in bold are found within the preporicilin linker region. The '-' symbol within the linker designate deleted amino acids.

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FIGURE 7B (P1)

Sequence of pAP309 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGA CTACAAGTTT				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTTGTGTAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAACGTTAGGTTTACTAAAGTCTTCGTCGTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

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FIGURE 7B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGGTCCTCTTGGCATGTGGGGACAACGAAATTTTAATGC
AGCAGTGTCAAACCAGGAGAACCGTACACCCCTGTTGCTTTAAATACG

951 TGATGTTTGTGGTGGCGGAGGGCCCATAGTGCGTATCGTAGGTGGAATG
ACTACAAACACCACCGCCTCCCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATCCTATCTCCTGACATCGTCACTTT

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FIGURE 7B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCA GTGTGTGTCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: pAP309

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

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FIGURE 7C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP309 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP-309 (MMP-9) linker:	A chain- C A P P S S Q F G P L G M W G Q R N F N A D V C G G G G -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '.' symbol within the linker designate deleted amino acids.

FIGURE 8A

Sequence of pAP313 (UPA) Linker Region

WT preprorocin linker

```

                    primer 313-3'
5' - GTAGTCGGCGGG-----TGTATGGATCCTGAG -3'
      * ***** *
-CTCATGGTGTATAGATGCGCACCTCCACCATCGTCACAGTTTCTTTGCTT | ATAAGGCCAGTGGTACCAATTTTAAATGCTGATGTTTGTATGGATCCTGAGCCCC-
-GAGTACCACATATCTACGCGTGGAGGTGGTAGCAGTGTCAAAGAAACGAA | TATTCGGTCCACCATGGTTTAAATATTAGGACTACAAACATACCTAGGACTCGGG-
      * ***** *
3' -TACCACATATCTACG-----GGTCCTGCT -5'
                    primer 313-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

```

pAP313 linker
(UPA variant)
CCAGGACGA | GTAGTCGGCGGG
GGTCCTGCT | CATCAGCCGCCCC

```

Note: Nucleotides in bold are found within the preprorocin linker region. The '.' symbol within the linker designate deleted nucleotides.

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10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTAA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTGTAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

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FIGURE 8B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTAAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGC-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACG-----

901 -----CCAGGACGAGTAGTCGGCGGG-----
-----GGTCCTGCTCATCAGCCGCC-----

951 -----TGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
-----ACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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FIGURE 8B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTGAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1807.

Sequence name: pAP313

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

Amino acid sequence Comparison of Mutant Preprorin Linker Region of PAP313 (UPA) to Wild Type

Note: Amino acids in bold are found within the preproreicin linker region. The ‘.’ symbol within the linker designate deleted amino acids.

FIGURE 9A

Sequence of pAP314 (UPA) Linker Region

WT preporicrin linker

```

                    primer 314-3'
5'- GTAGTCGGCGGG-----GGAGCGGGGGTTGTATGGATCCTGAG -3'
  * ***** *
  * **** *
  * **** *
-CTCATGGTGTATAGATCGGCACCTCCACCATCGTCACAGTTTCTTTGCTT | ATAAGGCCAGTGGTACCAAAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
-GAGTACCACATATCTACGCGTGGAGGTTGGTAGCAGTGTCAAAAGAAACGAA | TATTCCGGTCCACCATGGTTTAAATTAAGACTACAAACATACCTAGGACTCGGG-
  * ***** *
  * **** *
3' -TACCACATATCTACGCCCTCGGCCCCCA-----GGTCCTGCT -5'
                    primer 314-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

```

pAP314 linker
(UPA variant)
GGAGCGGGGGTCCAGGACGA | GTAGTCGGCGGGGGAGGCGGGGGT
CCTCCGCCCCCAGGTCCTGCT | CATCAGCCGCCCCCTCCGCCCCCA

```

Note: Nucleotides in bold are found within the preporicrin linker region. The '-' symbol within the linker designate deleted nucleotides.

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FIGURE 9B (P1)

Sequence of pAP314 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

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FIGURE 9B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGGGGT
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCGCCCCCA

901 -----CCAGGACGAGTAGTCGGCGGG-----GGAGG
-----GGTCCTGCTCATCAGCCGCC-----CCTCC

951 CGGGGGTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
GCCCCAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 9C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP314 (UPA) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP314 (UPA) linker:	A chain- C G G G G - - - P G R V V G G - - - G G G G C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

Sequence of pAP315 (UPA) Linker Region

WT preproricin linker

```

                    primer 315-3'
5' - CCAGGACGAGTAGTCGGCGGG-----TGATGGATCCTGAG -3'
      ** * * * *
-CTCATGGTGTATAGATGCGCACCTCCACCATCGTCACAGTTTTCTTTGCTT|ATAAGGCGCAGTGGTACCAAAATTTAAATGCTGATGTTTGTATGGATCCTGAGCCC-
-GAGTACCACATATCTACGGGTGGAGGTGTAGCAGTGTCAAAGAGAAACGAA|TATTCCGCTCACCATGGTTTAAAAATTACGACTACAAACATACCTAGGACTCGGG-
      * * * * *
3' -TACCACATATCTACG-----GGTCCTGCTCATCAGCGGCC -5'
                    primer 315-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

pAP315 linker
(UPA variant)
CCAGACGAGTAGTCGGCGG | CCAGACGAGTAGTCGGCGGG
GGTCCTGCTCATCAGCGGCC | GGTCCTGCTCATCAGCGCGCC

Note: Nucleotides in bold are found within the preprorion linker region. The ‘.’ symbol within the linker designate deleted nucleotides.

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FIGURE 10B (P1)

Sequence of pAP315 insert

```
          10          20          30          40          50
          |          |          |          |          |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGAATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTGCTTC
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
```

701 GATCTGCACCAGATCCTTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGC-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACG-----

901 CCAGGACGAGTAGTCGGCGGGCCAGGACGAGTAGTCGGCGGG-----
GGTCCTGCTCATCAGCCGCCCGGTCTGCTCATCAGCCGCC-----

951 -----TGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGAAATG
-----ACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTCACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

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FIGURE 10B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTGAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1828.

Sequence name: pAP315

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

Amino acid sequence Comparison of Mutant Preprorin Linker Region of PAP315 (UPA) to Wild Type

Note: Amino acids in bold are found within the preprorelin linker region. The '-' symbol within the linker designate deleted amino acids.

Note: Amino acids in bold are found within the preprorelin linker region. The '-' symbol within the linker designate deleted amino acids.

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FIGURE 11A**Sequence of pAP316 (MMP-9) Linker Region****WT preprorin linker**

primer 316-3'
 5'- ATTGCAGGCGCAGGAGGGGTAGTAGCGCGGGGATGTATGGATCCTGAG -3'
 ***** * ***** * * * * *
 -CTCATGGTGTATAGATGGGCACCTCCACCATCGTCACAGTTTCTTTGCTT|ATAAGGCCAGTGGTACCAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCCC-
 -GAGTACCACATATCTACGCGTGGAGGTGGTAGCAGTGTCAAAGAAACGAA|TATTCGGTCAACCATGGTTTAAATACGACTACAAACATACCTAGGACTCGGG-
 * ***** * * * * *
 3' -TACCACATATCTACGCTCCGCCCTGAGGTCCGCCCCCAGGCGTTCT -5'

1) PCR mutagenesis

2) Ligate with pVL1393

**pAP316 linker
(MMP-9 variant)**

GGAGCGGGGACTCCAGCGGGGTCCGCAAGGA|ATTGCAGGCGCAGGAGGGGGTAGTAGCGCGGGGGA
 CCTCCGCCCCCTGAGGTGCGCCCCCAGGCGTTCT|TAACGTCCCGTCCCTCCCCCATCATCGCCGCCCT

Note: Nucleotides in bold are found within the preprorin linker region. The '-' symbol within the linker designate deleted nucleotides.

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FIGURE 11B (P1)

Sequence of pAP316 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG				
	CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGA CTACAAGTTTTA				
451	CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACA ACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTGTAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCA ACT				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTT CAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT C				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

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FIGURE 11B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGGGGT
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCGCCCTGA

901 GGAGGCGGGGGTCCGCAAGGAATTGCAGGGCAGGGAGGGGGTAGTAGCGG
GGTCCGCCCCCAGGCGTTCCTTAACGTCCCGTCCCTCCCCCATCATCGCC

951 CGGGGGATGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
GCCCCCTACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 11C

Amino acid sequence Comparison of Mutant Preprorin Linker Region of PAP316 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP316 (MMP-9) linker:	A chain- C G G G S S G G G P Q G I A G Q G G S S G G G C M D P E -B chain

Note: Amino acids in bold are found within the preprorin linker region. The ‘.’ symbol within the linker designate deleted amino acids.

Sequence of pAP318 (MMP-9) Linker Region

WT preprorcin linker

5' - ATTGCAGGGCAGGATGAAGAGGATGCTGATGTTGTATG -3'
 primer 318-3'
 **** * ***** *****
 -CTCATGGTGTATAGATGGGCACCTCCACCATCGTCACAGTTTTCTTTGCTTATA | AGGCCAGTGGTACCCAAATTTTAATGCTGATGTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACGGCTGGAGGTGGTAGCAGTGTCAAAGAGAAACGAATAT | TCCGGTCACCATGGTTTAAATACGACTACAAACATACCTAGGACTCGGG-
 ***** ** *****
 3' - GGAGGTGGTAGCAGTCTCTCCAAGAGGGGTTTCCT -5'
 primer 318-5'

- 1) PCR mutagenesis
- 2) Ligate with pVL1393

GCACCTCCACCATCGTCAGGAGGTTCTCCGCAAGGA | ATTCAGGGGACGATGTAAGAGGATGCTGATGTT
CGTGGAGGTGGTAGCAGTCCTCCAGAGGSGGTTCCCT | TTAACGTCCCGTCTCTACTTCTCTTACGACTACAA

Note: Nucleotides in bold are found within the preprorin linker region. The '-' symbol within the linker designate deleted nucleotides.

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FIGURE 12B (P1)

Sequence of pAP318 insert

```
          10          20          30          40          50
          |          |          |          |          |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGTAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTTATAATTTGCATCCAAATGATTTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTGCTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
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FIGURE 12B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCGGAGGTTCTCCGCAAGGAATTGCAGGGCAGGATGAAGAGGAATGC
AGCAGCCTCCAAGAGGCGTTCCTTAACGTCCCGTCCTACTTCTCCTTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGGAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATCCTATCTCCTGACATCGTCACTTT

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FIGURE 12B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: pAP318

Note: Nucleotides in bold are found within the mutant preprorcin linker region. The '-' symbol within the linker region designate deleted nucleotides.

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FIGURE 12C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP318 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP318 (MMP-9) linker:	A chain- C A P P S S G G S P Q G I A G Q D E E D A D V C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

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FIGURE 13A**Sequence of pAP320 (UPA) Linker Region****WT preprorin linker**

```

                    primer 320-3'
5'- GTAGTCGGCGGG-----GGGGAGGCTGTATGGATCCTGAG -3'
  * ***** *
-CTCATGGTGTATAGATGCGCACCTCCACCATCGTCACAGTTTCTTTGCTT | ATAAGGCCAGTGGTACCAAAATTTTAATGCTGATGTTGTATGGATCCTGAGCCC-
-GAGTACCACATATCTACCGTGGAGGTGGTAGCAGTGTCAAAGAAACGAA | TATTCGGTCACCATGGTTTAAATACGACTACAAACATACCTAGGACTCGGG-
  * ***** *
3' -TACCACATATCTACGCCTCCGCCT-----GGTCCTGCT -5'
                    primer 320-5'

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1) PCR mutagenesis

2) Ligate with pVL1393

**pAP320 linker
(UPA variant)**

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GGAGGCGGACCAGGACGA | GTAGTCGGCGGGGGGAGGC
CCTCCGCCTGCTCCTGCT | CATCAGCCGCCGCCCTCCG

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Note: Nucleotides in bold are found within the preprorin linker region. The '-' symbol within the linker designate deleted nucleotides.

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FIGURE 13B (P1)

Sequence of pAP320 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTGCGGG				
	CGCCCACGGTGACACGTTTCGATGTGTTTGAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTGA				
451	CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTACGCGTGCTCTTAATCCATGTTGGCCT				

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FIGURE 13B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGA ---
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCGCCT ---

901 -----CCAGGACGAGTAGTCGGCGGG-----GG
-----GGTCCTGCTCATCAGCCGCC-----CC

951 GGGAGGCTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG
CCCTCCGACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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FIGURE 13B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTCTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1825.

Sequence name: pAP320

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 13C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP320 (UPA) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP320 (UPA) linker:	A chain- C G G G - - - - P G R V V G G - - - - G G G C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The ‘.’ symbol within the linker designate deleted amino acids.

Sequence of pAP321 (UPA) Linker Region

WT preprorcin linker

```

      primer 321-3'
    5'- GTAGTCGGCGG-----GGAGGTGTATGGATCCTGAG -3'
          * ***** *           ** **
            CTCATGGTGTATAGATGCACACTCCACCATCGTCACAGTTTCTTTTGCTT | ATAAGGCCAGTGGTAGCAAAATTTAATGCTGATGTTTTGTATGGATCCTGAGCCC-
            GAGPACCACATATCTACGGCTGGAGGTGTAGCAGTGTCAAAGAACAACGA   TATTCCGGTCAACCATGGTTTAAAAATTACGACTACAAACATACCTAGGACTCGGG-
                                * ***                      * **** **
        3' -TACCACATATCTACGCCTCG-----GGTCCTGCT -5'
                                     primer 321-5'

```

- 1) PCR mutagenesis
- 2) Ligate with pVL1393

pAP321 linker
(UPA variant)
GGAGGCCCCAGGACGA GTAGTCGGCGGGGGAGGC
CCTCCGGGTCTCTGCT CATCAGCGCGCCCCCTCCG

Note: Nucleotides in bold are found within the preprorin linker region. The ‘.’ symbol within the linker designate deleted nucleotides.

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FIGURE 14B (P1)

Sequence of pAP321 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTTA				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAAGTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCCTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

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FIGURE 14B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGC-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCG-----

901 -----CCAGGACGAGTAGTCCGGCGGG-----
-----GGTCCTGCTCATCAGCCGCC-----

951 -GGAGGCTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCGAAATG
-CCTCCGACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACCAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT

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FIGURE 14B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1819.

Sequence name: pAP321

Note: Nucleotides in bold are found within the mutant preprorycin linker region. The '-' symbol within the linker region designate deleted nucleotides.

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FIGURE 14C**Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP321 (UPA) to Wild Type**

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP321 (UPA) linker:	A chain- C G G - - - - - P G R V V G G - - - - - G G C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 15A

Sequence of pAP322 (UPA) Linker Region

WT preprorin linker

```

                    primer 322-3'
5'- GTAGTCGGCGG-----GGCTGTATGGATCCTGAG -3'
      * ***** **
-CTCATGGTGTATAGATGGCGACCTCCACCATCGTCACAGTTTCTTTGCTT | ATAAGGCCAGTGGTACCAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
-GAGTACCACATATCTACGGGTGGAGGTGGTAGCAGTGTCAAAAGAAACGAA | TATTCGGTCCACCATGGTTTAAATTACGACTACAAACATACCTAGGACTCGGG-
      * ***** **
3' -TACCACATATCTACGCCT-----GGTCCTGCT -5'
                    primer 322-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

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pAP322 linker
(UPA variant)
GGACCAGGACGA | GTAGTCGGCGGGGGC
CCTGGTCCTGCT | CATCAGCCGCCCCCG

```

Note: Nucleotides in bold are found within the preprorin linker region. The '-' symbol within the linker designate deleted nucleotides.

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FIGURE 15B (P1)

Sequence of pAP322 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGT	TTTGGATCCACCTCAGGGTGGTCTTT	CACATTAG		
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGT	TATGGGTTAATATTTGAAATGGTGT			
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTT	CGCGG			
	CGCCACGGTGACACGTTTCGATGTGTTT	GAAATAGTCTCGACAAGCGCC			
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGT	TGCCAA			
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTTGTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

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FIGURE 15B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGA-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCT-----

901 -----CCAGGACGAGTAGTCGGCGGG-----
-----GGTCCTGCTCATCAGCCGCC-----

951 ----GGCTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
----CCGACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTT CAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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FIGURE 15B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTAGAT
AGTTCCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCTGTGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1813.

Sequence name: pAP322

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

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FIGURE 15C**Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP322 (UPA) to Wild Type**

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP322 (UPA) linker:	A chain- C G - - - - - P G R V V G G - - - - - G C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 16A

Sequence of pAP323 (MMP-9) Linker Region

WT preprorin linker

primer 323-3'
 5' - ATTCAGGGCAG---GGGGGTAGTAGCGGCGGGGATGTATGGATCCTGAG -3'
 ***** ** ** ** **
 -CTCATGGTGTATAGATGGGACCTCCACCATCGTCACAGTTTCTTTGCTT|ATAAGGCCAGTGGTACCAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACGGGTGGAGGTGGTAGCAGTGTCAAAGAAACGAA|TATTCGGTCAACCATGGTTTAAATTTAGGACTACAAACATACCTAGGACTCGGG-
 * ***** ** ** *
 3' -TACCACATATCTACGCCTCCGCCCTGAGGT---CCCCCAGGGGTTCTCT -5'
 primer 323-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP323 linker
(MMP-9 variant)

GGAGGGGGGACTCCAGGGGTCCGCAAGGA|ATTGCAGGCGAGGGGGGTAGTAGCGGCGGGGGA
 CCTCGGCCCTGAGGTCCCCCAGGCGTCTCT|TAACGTCCCGTCCCCCATCATCGCCGCCCT

Note: Nucleotides in bold are found within the preprorin linker region. The '.' symbol within the linker designate deleted nucleotides.

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FIGURE 16B (P1)

Sequence of pAP323 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGCGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC				
	GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTACGCGTGCTCTTAATCCATGTTGGCCT				

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGGACT
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCGCCCTGA

901 CCAGGG---GGTCCGCAAGGAATTGCAGGGCAG---GGGGGTAGTAGCGG
GGTCCC---CCAGGCGTTCCCTTAACGTCCCGTC---CCCCATCATCGCC

951 CGGGGGATGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG
GCCCCCTACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

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FIGURE 16C**Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP323 (MMP-9) to Wild Type**

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP323 (MMP-9) linker:	A chain- C G G G S S - G G P Q G I A G Q - G G S S G G G C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

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FIGURE 17A**Sequence of pAP324 (MMP-9) Linker Region****WT preprorin linker**

primer 324-3'
 5' - ATTGCAGGGCAG-----GGTAGTAGCGGGGGGATGTATGGATCCTGAG -3'

 ** ** ** ** ** ** **
 -CTCATGGGTATAGATGCGCACCTCCACCATCGTCACAGTTTCTTTGCTT|ATAAGGCCAGTGGTACCAAAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACGCGTGGAGTGGTAGCAGTGTCAAAGAAACGAA|TATTCCGGTCACCATGGTTTAAATTTACGACTACAAACATACCTAGGACTCGGG-
 * ***** ** *
 3' -TACCACATATCTACGCCTCCGCCCTGAGGT-----CCAGGCGTTCTCT -5'
 primer 324-5'

1) PCR mutagenesis

2) Ligate with pVL1393

AP324 linker**(MMP-9 variant)**

GGAGGGGGACTCCAGGTCCGCAAGGA|ATTGCAGGGCAGGTTAGTAGCGGGGGGGA
 CCTCCGCCCTGAGTCCAGGCGTTCTCT|TAACGTCCCGTCCCATCATCGCCGCCCTCT

Note: Nucleotides in bold are found within the preprorin linker region. The '-' symbol within the linker designate deleted nucleotides.

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FIGURE 17B (P1)

Sequence of pAP324 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG				
	CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTIONAAGTTT				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC				
	GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACT				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

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FIGURE 17B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTAAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGGACT
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCGCCCTGA

901 CCA-----GGTCCGCAAGGAATTGCAGGGCAG-----GGTAGTAGCGG
GGT-----CCAGGCGTTCCTTAACGTCCCGTC-----CCATCATCGCC

951 CGGGGGATGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
GCCCCCTACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTACATACCTATCTCCTGACATCGTCACTTT

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FIGURE 17B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1843.

Sequence name: pAP324

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 17C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP324 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP324 (MMP-9) linker:	A chain- C G G G S S - - G P Q G I A G Q - - G S S G G G C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

Sequence of pAP325 (MMP-9) Linker Region

WT preprorcin linker

primer 325-3',
 5' - ATTGCAAGGCAG-----AGTAGCGGGGGGATGTATGGATCCTGAG -3',

 ** ** ** ** **
 -CTCATGGTGTATAGATGCGCACCTCCACCATCGTCCAGTTTCTTTGCTT | ATAAGGCCAGTGGTACCAAAATTTAATGCTGATGTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACGGTGGAGGTGTAGCAGTGTCAAAGAAACGAA | TATTCGGTCAACCATGGTTTAAAAATTACGACTACAAACATACCTAGGACTCGGG-
 * ***** ** * * *****
 3' -TACCACATATCTACGCCCTCGGCCCTGAGGT-----GGCGTTTCCT -5',
 primer 325-5'

- 1) PCR mutagenesis
- 2) Ligate with pVL1393

pAP325 linker
(MMP-9 variant)
GGAGGCGGGACTCCACCGCAAGGA | ATTGCAGGGCAGAGTAGCGGCGGGGGA
CCTCCGCGCTGAGGTGGCGTTCCT | TAACGTCCCGTCTCATCGCGCGCCCCCT

Note: Nucleotides in bold are found within the preprorion linker region. The ‘.’ symbol within the linker designate deleted nucleotides.

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FIGURE 18B (P1)

Sequence of pAP325 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGCG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

FIGURE 18B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGGACT
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCGCCCTGA

901 CCA-----CCGCAAGGAATTGCAGGGCAG-----AGTAGCGG
GGT-----GGCGTTCCTTAACGTCCCGTC-----TCATCGCC

951 CGGGGGATGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG
GCCCCCTACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGA.CATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTGTTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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FIGURE 18B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1837.

Sequence name: pAP325

Note: Nucleotides in bold are found within the mutant preprorin linker region. The '-' symbol within the linker region designate deleted nucleotides.

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FIGURE 18C**Amino acid sequence Comparison of Mutant Preproricein Linker Region of PAP325 (MMP-9) to Wild Type**

Wild type ricin linker:	A chain- C A P P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP325 (MMP-9) linker:	A chain- C G G G S S - - - P Q G I A G Q - - - S S G G G C M D P E -B chain

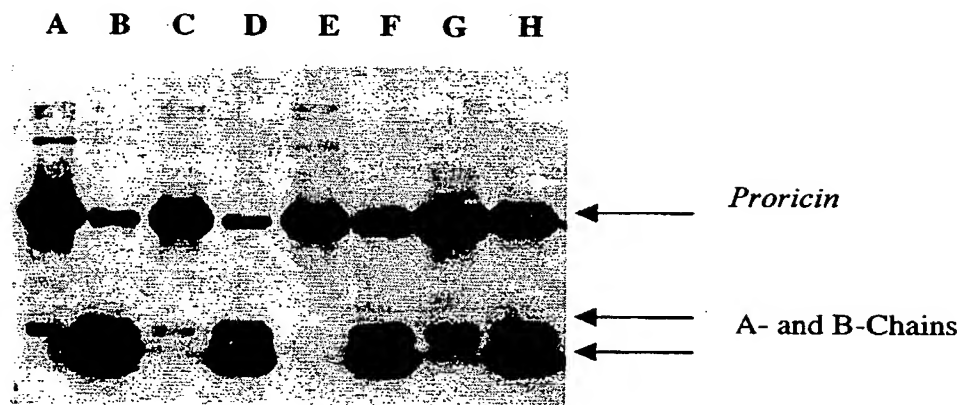
Note: Amino acids in bold are found within the preproricein linker region. The '-' symbol within the linker designate deleted amino acids.

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FIGURE 19

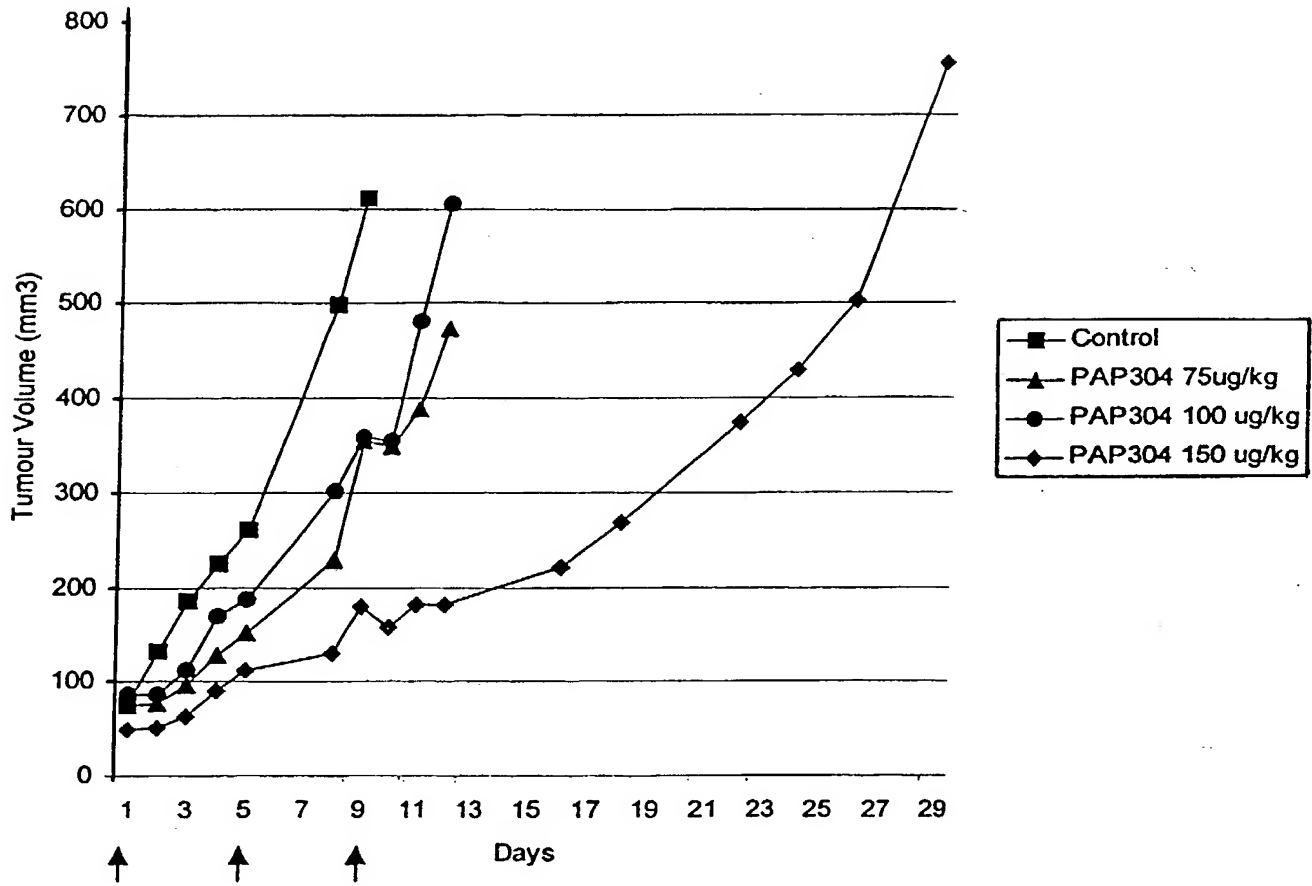
Cleavage of Proricin Variants by Matrix Metalloprotease-9



- A** PAP220 (500 ng)
B PAP220 (500 ng) digested with 100 ng MMP9
C PAP323 (500 ng)
D PAP323 (500 ng) digested with 100 ng MMP9
E PAP324 (500 ng)
F PAP324 (500 ng) digested with 100 ng MMP9
G PAP325 (500 ng)
H PAP325 (500 ng) digested with 100 ng MMP9

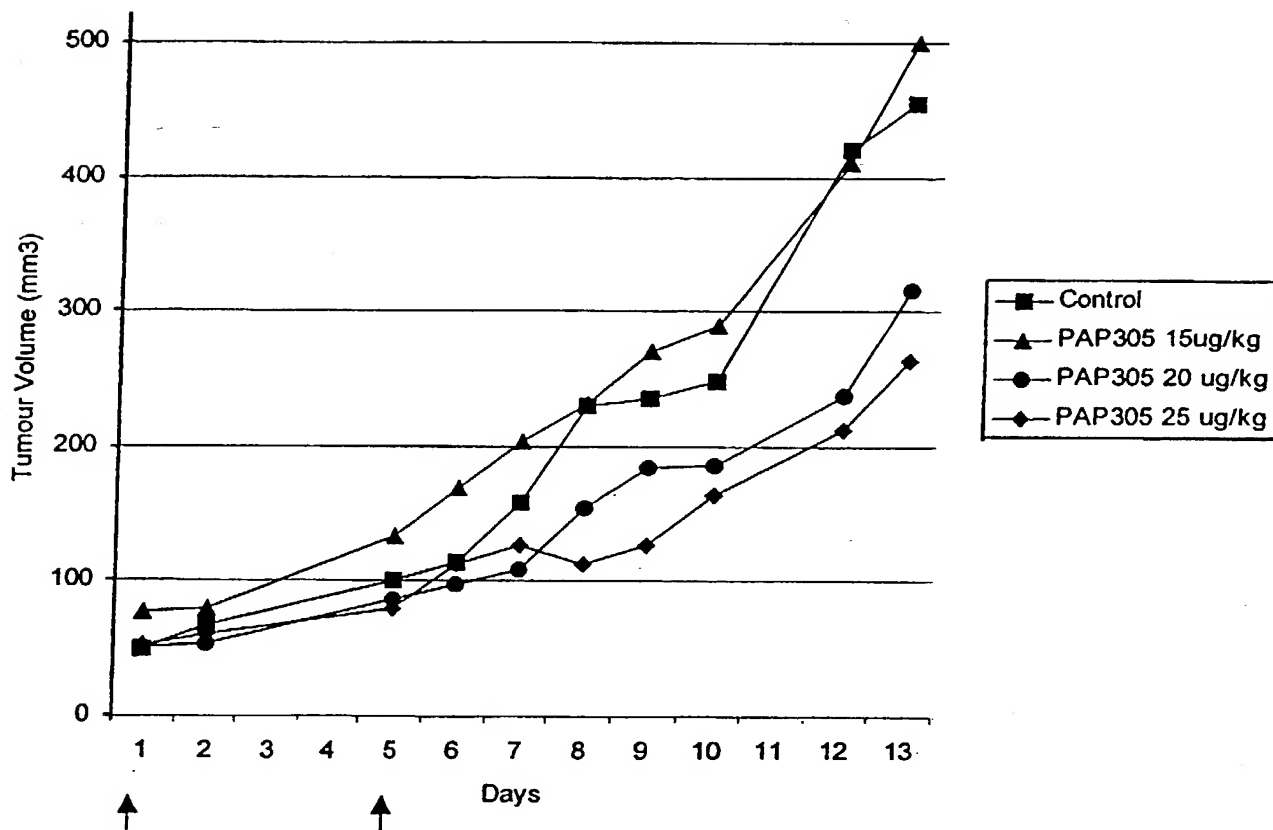
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FIGURE 20

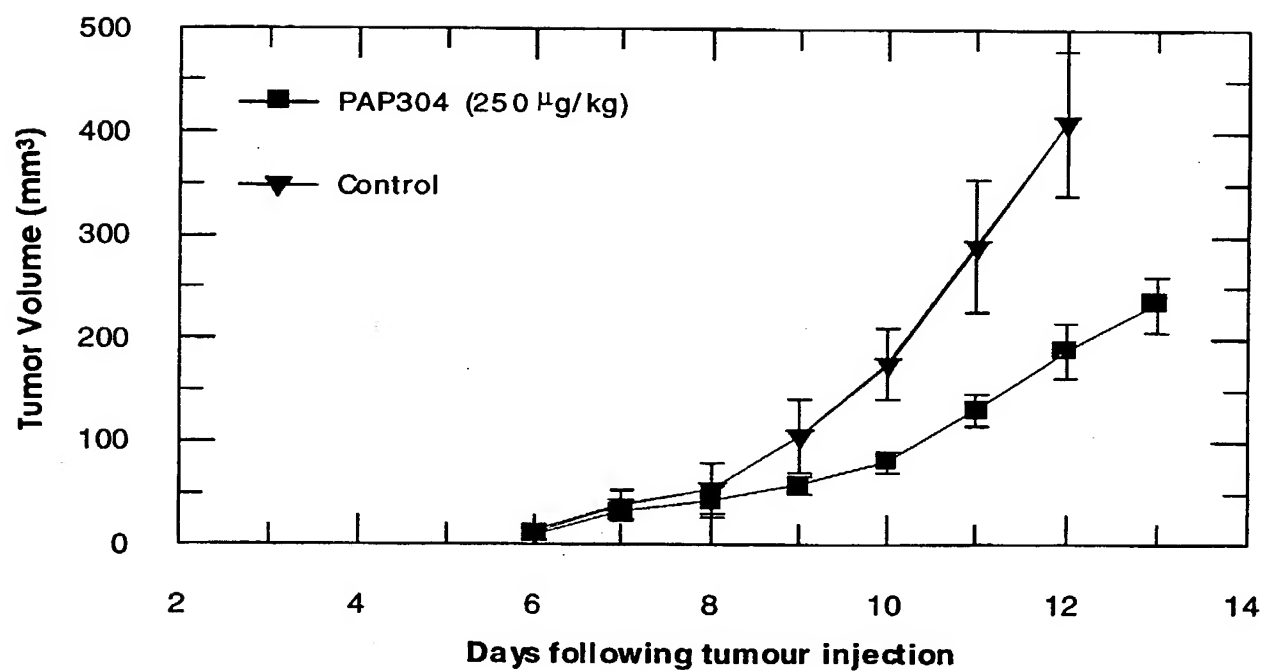


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FIGURE 21



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FIGURE 22

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**DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN
APPLICATION DATA SHEET (37 CFR 1.76)**

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or☒ Application No. PCT/CA00/01162, filed on October 4, 2000,☐ as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)Inventor one: Curtis BraunSignature: Citizen of: CanadaInventor two: Admir PuracSignature: Citizen of: CanadaInventor three: Thor BorgfordSignature: Citizen of: Canada

Inventor four: _____

Signature: _____

Citizen of: _____

☐ Additional inventors are being named on _____ additional form(s) attached hereto.

Burden Hour Statement: This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is used by the public to file (and the PTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This form is estimated to take 1 minute to complete. This time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.